NPC1L1 (NPC3) AND METHODS OF IDENTIFYING LIGANDS THEREOF

SPECIFICATION

The invention claimed herein was made on behalf of Merck & Co., Inc. and Schering-Plough Corporation, parties to a joint research agreement that was in effect on or before the date the claimed invention was made.

This application claims priority to Serial No. 60/537,341, filed January 16, 2004.

FIELD OF THE INVENTION

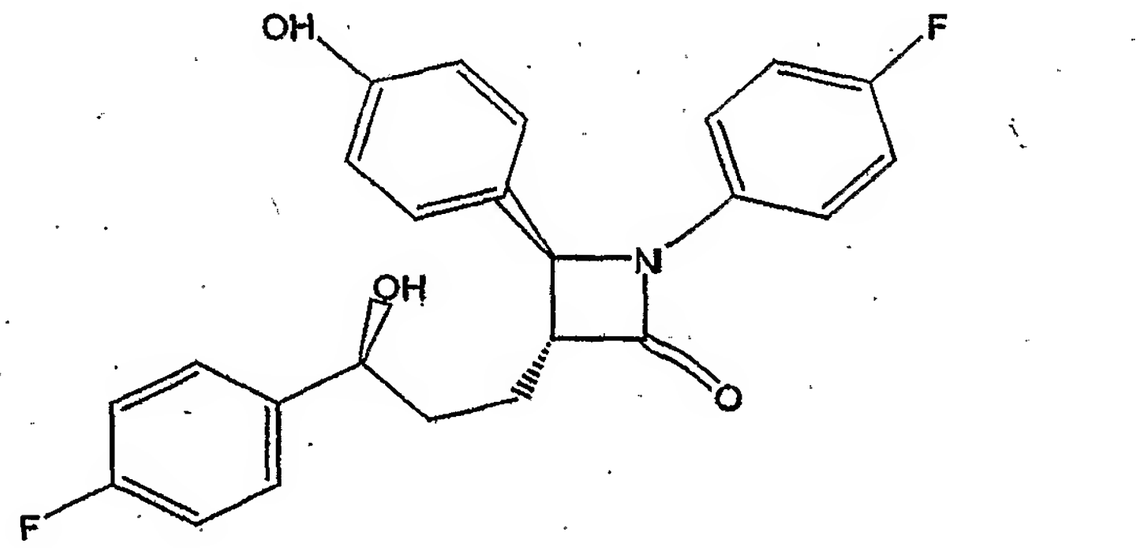
The present invention includes NPC1L1 polypeptides and polynucleotides which encode the polypeptides, methods of use and methods of identifying modulators and ligands thereof.

BACKGROUND OF THE INVENTION

A factor leading to development of vascular disease, a leading cause of death in industrialized nations, is elevated serum cholesterol. It is estimated that 19% of Americans between the ages of 20 and 74 years of age have high serum cholesterol. The most prevalent form of vascular disease is arteriosclerosis, a condition associated with the thickening and hardening of the arterial wall. Arteriosclerosis of the large vessels is referred to as atherosclerosis. Atherosclerosis is the predominant underlying factor in vascular disorders such as coronary artery disease, aortic aneurysm, arterial disease of the lower extremities and cerebrovascular disease.

Cholesteryl esters are a major component of atherosclerotic lesions and the major storage form of cholesterol in arterial wall cells. Formation of cholesteryl esters is also a step in the intestinal absorption of dietary cholesterol. Thus, inhibition of cholesteryl ester formation and reduction of serum cholesterol can inhibit the progression of atherosclerotic lesion formation, decrease the accumulation of cholesteryl esters in the arterial wall, and block the intestinal absorption of dietary cholesterol.

The regulation of whole-body cholesterol homeostasis in mammals and animals involves the regulation of intestinal cholesterol absorption, cellular cholesterol trafficking, dietary cholesterol and modulation of cholesterol biosynthesis, bile acid biosynthesis, steroid biosynthesis and the catabolism of the cholesterol-containing plasma lipoproteins. Regulation of intestinal cholesterol absorption has proven to be an effective means by which to regulate serum cholesterol levels. For example, a cholesterol absorption inhibitor, ezetimibe (



), has been shown to be effective in this regard. A pharmaceutical composition containing ezetimibe is commercially available from Merck/Schering-Plough Pharmaceuticals, Inc. under the trade name Zetia®. Identification of a gene target through which ezetimibe acts is important to understanding the process of cholesterol absorption and to the development of other, novel absorption inhibitors. The present invention addresses this need by providing a rat and a mouse homologue of human NPC1L1 (also known as NPC3; Genbank Accession No. AF192522; Davies, *et al.*, (2000) Genomics 65(2): 137-45 and Ioannou, (2000) Mol. Genet. Metab.71(1-2): 175-81), an ezetimibe target.

NPC1L1 is an N-glycosylated protein comprising a YQRL (SEQ ID NO: 38) motif (i.e., a *trans*-golgi network to plasma membrane transport signal; see Bos, *et al.*, (1993) EMBO J. 12: 2219-2228; Humphrey, *et al.*, (1993) J. Cell. Biol. 120: 1123-1135; Ponnambalam, *et al.*, (1994) J. Cell. Biol. 125: 253-268 and Rothman, *et al.*, (1996) Science 272: 227-234) which exhibits limited tissue distribution and gastrointestinal abundance. Also, the human *NPC1L1* promoter includes a Sterol Regulated Element Binding Protein 1 (SREBP1) binding consensus sequence (Athanikar, *et al.*, (1998) Proc. Natl. Acad. Sci. USA 95: 4935-4940; Ericsson, *et al.*, (1996) Proc. Natl. Acad. Sci. USA 93: 945-950; Metherall, *et al.*, (1989) J. Biol. Chem. 264: 15634-15641; Smith, *et al.*, (1990) J. Biol. Chem. 265:

2306-2310; Bennett, *et al.*, (1999) J. Biol. Chem. 274: 13025-13032 and Brown, *et al.*, (1997) Cell 89: 331-340). NPC1L1 has 42% amino acid sequence homology to human NPC1 (Genbank Accession No. AF002020), a receptor responsible for Niemann-Pick C1 disease (Carstea, *et al.*, (1997) Science 277: 228-231). Niemann-Pick C1 disease is a rare genetic disorder in humans which results in accumulation of low density lipoprotein (LDL)-derived unesterified cholesterol in lysosomes (Pentchev, *et al.*, (1994) Biochim. Biophys. Acta. 1225: 235-243 and Vanier, *et al.*, (1991) Biochim. Biophys. Acta. 1096: 328-337). In addition, cholesterol accumulates in the *trans*-golgi network of npc1$^-$ cells, and relocation of cholesterol, to and from the plasma membrane, is delayed. NPC1 and NPC1L1 each possess 13 transmembrane spanning segments as well as a sterol-sensing domain (SSD). Several other proteins, including HMG-CoA Reductase (HMG-R), Patched (PTC) and Sterol Regulatory Element Binding Protein Cleavage-Activation Protein (SCAP), include an SSD which is involved in sensing cholesterol levels possibly by a mechanism which involves direct cholesterol binding (Gil, *et al.*, (1985) Cell 41: 249-258; Kumagai, *et al.*, (1995) J. Biol. Chem. 270: 19107-19113; Hua, *et al.*, (1996) Cell 87: 415-426; and Radhakrishnan, A., *et al.*, "Direct binding of cholesterol to the purified membrane region of SCAP: Mechanism for a sterol-sensing domain," *Mol. Cell* 15, 259-268 (2004)).

SUMMARY OF THE INVENTION

The present invention is based on the discovery that NPC1L1 is the target through which ezetimibe acts, and consequently plays a critical role in the regulation of sterol and 5α-stanol intestinal transport and absorption, *e.g.* cholesterol absorption. Accordingly, this invention provides for the use of NPC1L1 in an assay for identifying ligands that block NPC1L1-mediated sterol and 5α-stanol intestinal transport. The present invention provides methods for identifying ligands of NPC1L1 which involve contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and a candidate compound, and determining whether the candidate compound binds to NPC1L1. The modulation of the binding of the substituted 2-azetidinone to NPC1L1 by the binding of the candidate compound to NPC1L1 indicates that the candidate compound is a ligand that binds to NPC1L1 and is an inhibitor of sterol and 5α-stanol absorption.

The present invention also provides a method for identifying a ligand of NPC1L1 comprising contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and measuring the binding of detectably labeled substituted 2-azetidinone to NPC1L1 in the presence and absence of a candidate compound, wherein decreased binding of the detectably labeled substituted 2-azetidinone to the NPC1L1 in the presence of the candidate compound indicates that said candidate compound is a ligand of NPC1L1 and is an inhibitor of sterol and 5α-stanol absorption.

The present invention also provides for a method for identifying a compound that inhibits intestinal sterol or 5α-stanol absorption mediated by NPC1L1 involving contacting NPC1L1 with a detectably labeled ligand and the candidate compound and determining whether the candidate compound binds to NPC1L1, wherein binding of said candidate compound to NPC1L1 modulates binding of said ligand to NPC1L1, wherein said modulation indicates that the candidate compound is an intestinal sterol or 5α-stanol absorption inhibitor.

The present invention provides methods for identifying an ligand of NPC1L1 comprising (a) contacting a host cell (*e.g.*, human embryonic kidney (HEK) 293 cells, chinese hamster ovary (CHO) cell, a J774 cell1, a macrophage cell or a Caco2 cell) expressing a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface, in the presence of a known amount of a detectably labeled (*e.g.*, with ^{3}H, ^{14}C, ^{125}I, ^{35}S or fluorescence labeling) substituted azetidinone (*e.g.*, ezetimibe), with a sample to be tested for the presence of an NPC1L1 ligand; and (b) measuring the amount of detectably labeled substituted azetidinone (*e.g.*, ezetimibe) specifically bound to the polypeptide; wherein an NPC1L1 ligand in the sample is identified by measuring substantially reduced binding of the detectably labeled substituted azetidinone (*e.g.*, ezetimibe) to the polypeptide, compared to what would be measured in the absence of such a ligand.

Another method for identifying an ligand of NPC1L1 is also provided. The method comprises (a) placing, in an aqueous suspension, a plurality of support particles, impregnated with a fluorescer (*e.g.*, yttrium silicate, yttrium oxide, diphenyloxazole and polyvinyltoluene), to which a host cell (*e.g.*, chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a

polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface are attached; (b) adding, to the suspension, a radiolabeled (*e.g.*, with ^{3}H, ^{14}C or ^{125}I) substituted azetidinone (*e.g.*, ezetimibe) and a sample to be tested for the presence of a ligand, wherein the radiolabel emits radiation energy capable of activating the fluorescer upon the binding of the substituted azetidinone (*e.g.*, ezetimibe) to the polypeptide to produce light energy, whereas radiolabeled substituted azetidinone (*e.g.*, ezetimibe) that does not bind to the polypeptide is, generally, too far removed from the support particles to enable the radioactive energy to activate the fluorescer; and (c) measuring the light energy emitted by the fluorescer in the suspension; wherein an NPC1L1 ligand in the sample is identified by measuring substantially reduced light energy emission, compared to what would be measured in the absence of such a ligand.

Also provided is a method for identifying a ligand of NPC1L1 comprising (a) contacting a host cell (*e.g.*, Chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a polypeptide comprising an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface with detectably labeled (*e.g.*, with ^{3}H, ^{14}C or ^{125}I) sterol (*e.g.*, cholesterol) or 5α-stanol and with a sample to be tested for the presence of an ligand; and (b) measuring the amount of detectably labeled sterol (*e.g.*, cholesterol) or 5α-stanol in the cell; wherein an NPC1L1 antagonist in the sample is identified by measuring substantially reduced detectably labeled sterol (*e.g.*, cholesterol) or 5α-stanol within the host cell, compared to what would be measured in the absence of such an antagonist and wherein an NPC1L1 agonist in the sample is identified by measuring substantially increased detectably labeled sterol (*e.g.*, cholesterol) or 5α-stanol within the host cell, compared to what would be measured in the absence of such an agonist.

The present invention includes methods for inhibiting NPC1L1-mediated intestinal sterol (*e.g.*, cholesterol) or 5α-stanol uptake, in a subject, by administering a substance identified by the screening methods described herein to the subject. Such substances include compounds such as small molecule antagonists of NPC1L1 other than ezetimibe. Also contemplated are methods for antagonizing NPC1L1-mediated sterol (*e.g.*, cholesterol) or 5α-stanol absorption by administering anti-NPC1L1 antibodies. NPC1L1-mediated absorption of sterol (*e.g.*, cholesterol) or

5α-stanol can also be antagonized by any method which reduces expression of NPC1L1 in an organism. For example, NPC1L1 expression can be reduced by introduction of anti-sense *NPC1L1* mRNA into a cell of an organism or by genetic mutation of the *NPC1L1* gene in an organism (*e.g.*, by complete knockout, disruption, truncation or by introduction of one or more point mutations).

Also included in the present invention is a mutant transgenic mammal (*e.g.*, mouse, rat, dog, rabbit, pig, guinea pig, cat, horse), preferably a mouse comprising a homozygous or heterozygous mutation (*e.g.*, disruption, truncation, one or more point mutations, knock out) of endogenous, chromosomal *NPC1L1* wherein, preferably, the mouse does not produce any functional NPC1L1 protein. Preferably, the mutant mouse, lacking functional NPC1L1, exhibits a reduced level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption and/or a reduced level of serum sterol (*e.g.*, cholesterol) or 5α-stanol and/or a reduced level of liver sterol (*e.g.*, cholesterol) or 5α-stanol as compared to that of a non-mutant mouse comprising functional NPC1L1. Preferably, in the mutant mouse chromosome, the region of *NPC1L1* (SEQ ID NO: 45) deleted is from nucleotide 790 to nucleotide 998. In one embodiment, *NPC1L1* (SEQ ID NO: 11) is deleted from nucleotide 767 to nucleotide 975. Any offspring or progeny of a parent *NPC1L1* mutant mouse (i.e., *npc1l1*) of the invention which has inherited an *npc1l1* mutant allele is also part of the present invention.

The scope of the present invention also includes a method for screening a sample for an intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption antagonist comprising (a) feeding a sterol (*e.g.*, cholesterol) or 5α-stanol-containing substance (*e.g.*, comprising radiolabeled cholesterol, such as ^{14}C-cholesterol or ^{3}H-cholesterol) to a first and second mouse comprising a functional *NPC1L1* gene and to a third, mutant mouse lacking a functional *NPC1L1*; (b) administering the sample to the first mouse comprising a functional *NPC1L1* but not to the second mouse; (c) measuring the amount of sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the intestine of said first, second and third mouse (*e.g.*, by measuring serum cholesterol); and (d) comparing the levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in each mouse; wherein the sample is determined to contain the intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption antagonist when the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the first mouse and third mouse are

less than the amount of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the second mouse.

The present invention also encompasses a kit comprising (a) a substituted azetidinone (*e.g.*, ezetimibe) in a pharmaceutical dosage form (*e.g.*, a pill or tablet comprising 10 mg substituted azetidinone (*e.g.*, ezetimibe)); and (b) information, for example in the form of an insert, indicating that NPC1L1 is a target of ezetimibe. The kit may also include simvastatin in a pharmaceutical dosage form (*e.g.*, a pill or tablet comprising 5 mg, 10 mg, 20 mg, 40 mg or 80 mg simvastatin). The simvastatin in pharmaceutical dosage form and the ezetimibe in pharmaceutical dosage form can be associated in a single pill or tablet or in separate pills or tablets.

The present invention also provides any isolated mammalian cell (*e.g.*, isolated mouse cell, isolated rat cell or isolated human cell) which lacks a gene which encodes or can produce a functional NPC1L1 polypeptide. The isolated cell can be isolated from a mutant mouse comprising a homozygous mutation of endogenous, chromosomal *NPC1L1* wherein the mouse does not produce any functional NPC1L1 protein. Further, the mutation can be in a gene which when un-mutated encodes an amino acid sequence of SEQ ID NO: 12 (*e.g.*, comprising a nucleotide sequence of SEQ ID NO: 11). The cell can be isolated or derived from duodenum, gall bladder, liver, small intestine or stomach tissue. The cell can be an enterocyte.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows an equilibrium saturation binding plot exhibiting the binding of ^{3}H-EZE-glucuronide to rhesus brush border membrane (BBM) vesicles. Observed total binding (Total) is shown as open circles; nonspecific binding (NS) as triangles, and specific binding (S-bind) as solid circles.

Figure 1B shows a scatchard analysis of ^{3}H-EZE-glucuronide binding to rhesus brush border membrane vesicles.

Figure 2A shows an equilibrium saturation binding plot exhibiting the binding of ^{3}H-EZE-glucuronide (1) to rat brush border membrane vesicles. Observed total binding (open circles) and nonspecific binding (triangles), determined in the presence of 100 μM unlabeled ezetimibe glucuronide, are included; specific binding (solid circles) was assessed from the difference between total and nonspecific binding.

Binding was measured at 2.5 mg protein/ml in a volume of 100 µl after 1 hour incubation. Data were fit by nonlinear regression as described in Methods.

Figure 2B shows scatchard analysis of ^{3}H-EZE-glucuronide binding to rat brush border membrane vesicles. The binding data identify a single high-affinity site with K_D = 542 nM and Bmax = 20.7 pmol/mg protein.

Figure 3A shows association kinetic analysis of ^{3}H-EZE-glucuronide in rat brush border membrane vesicles. Conditions were 25 nM of **1** and 3 mg/ml protein at 25°C. The second-order rate constant k_{on} (0.55 x 10^{-4} M^{-1} s^{-1}) was calculated from k_{obs} (0.004 s^{-1}) as described in Methods.

Figure 3B shows dissociation kinetic analysis of ^{3}H-EZE-glucuronide **1** in rat brush border membrane vesicles. After the complex was formed by incubating 25 nM of **1** and 3 mg/ml protein for 1 hour, dissociation was initiated by competition with 100 µM unlabeled ezetimibe glucuronide. The curve is theoretical for k_{off} = 0.0024 s^{-1}.

Figure 4A shows association kinetic analysis of ^{3}H-EZE-glucuronide in rhesus brush border membrane vesicles.

Figure 4B shows dissociation kinetic analysis of ^{3}H-EZE-glucuronide in rhesus brush border membrane vesicles.

Figure 5 shows the results of a binding assay where ^{3}H-EZE-glucuronide is dissociated by EZE-glucuronide and compound **2** from rhesus (A) and rat (B) brush border membrane vesicles.

Figure 6 shows the results of a binding assay where ^{35}S-**2** is dissociated by EZE-glucuronide and **2** from mouse brush border membrane vesicles.

Figure 7 shows the distribution of ^{3}H-EZE-glucuronide binding to rhesus (A) and rat (B) brush border membranes prepared from various portions of rhesus (A) and rat (B) intestinal tissue.

Figure 8 shows the results of a binding assay where ^{35}S-**2** is dissociated by EZE-glucuronide and various analogs from CHO cells transfected with rat NPC1L1.

Figure 9 shows the results of a binding assay where ^{35}S-**2** is dissociated by EZE-glucuronide and various analogs from CHO cells transfected with human NPC1L1.

Figure 10 shows the binding of ^{35}S-**2** to brush border membrane vesicles prepared from wild type (A) and *NPC1L1* knockout (-/-) mice.

Figure 11 shows the results of a binding assay where ^{35}S-**2** is dissociated by compound **2** from mouse wild type and *NPC1L1* knockout (-/-) brush border membrane vesicles.

Figure 12A shows equilibrium determination of K_D for ezetimibe glucuronide by competition of unlabeled compound against **1** in rat enterocyte brush border membranes. Membranes (1.5 mg/ml protein) were incubated with 1 (50 nM) and the indicated concentrations of ezetimibe glucuronide for 1 hour to ensure equilibrium. K_D at equilibrium is 600 nM. Figure 12B shows the corresponding measurement for rhesus monkey, which were conducted between 0.5 and 1.25 mg/ml protein and 22-50 nM 1, with incubation time of more than 3 hours. K_D at equilibrium is 38.6 nM.

Figure 13 shows the expression of NPClL1 in HEK-293 cells using Western blot analysis (Panel 1) and immunofluorescence (Panel 2).

Figure 14A shows binding of ^{3}H-ezetimibe glucuronide to enterocyte brush border membranes from wild type mice and NPC1L1 deficient mice in the presence of detergent. Figure 14B shows competition studies of unlabeled ezetimibe glucuronide against labeled ezetimibe glucuronide.

Figure 15 shows the effect of detergents, taurocholate and digitonin, on [^{3}H]ezetimibe glucuronide binding.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes NPC1L1 polypeptides from rat, human and mouse, along with polynucleotides encoding the respective polypeptides. Preferably, the rat NPC1L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 2, the human NPC1L1 comprises the amino acid sequence set forth in SEQ ID NO: 4 and the mouse NPC1L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 12. The rat *NPC1L1* polynucleotide of SEQ ID NO: 1 or 10 encodes the rat NPC1L1 polypeptide. The human *NPC1L1* polynucleotide of SEQ ID NO: 3 encodes the human NPC1L1 polypeptide. The mouse *NPC1L1* polynucleotide of SEQ ID NO: 11 or 13 encodes the mouse NPC1L1 polypeptide.

The present invention includes any isolated polynucleotide or isolated polypeptide comprising a nucleotide or amino acid sequence referred to, below, in Table 1.

Table 1. Polynucleotides and Polypeptides of the Invention.

Polynucleotide or Polypeptide	Sequence Identifier
Rat *NPC1L1* polynucleotide	SEQ ID NO: 1
Rat *NPC1L1* polypeptide	SEQ ID NO: 2
Human *NPC1L1* polynucleotide	SEQ ID NO: 3
Human *NPC1L1* polypeptide	SEQ ID NO: 4
Rat *NPC1L1* expressed sequence tag 603662080F1 (partial sequence)	SEQ ID NO: 5
Rat *NPC1L1* expressed sequence tag 603665037F1 (partial sequence)	SEQ ID NO: 6
Rat *NPC1L1* expressed sequence tag 604034587F1 (partial sequence)	SEQ ID NO: 7
EST 603662080F1 with downstream sequences added	SEQ ID NO: 8
EST 603662080F1 with upstream and downstream sequences added	SEQ ID NO: 9
Back-translated polynucleotide sequence of rat NPC1L1	SEQ ID NO: 10
Mouse NPC1L1 polynucleotide	SEQ ID NO: 11
Mouse NPC1L1 polypeptide	SEQ ID NO: 12
Back-translated polynucleotide sequence of mouse NPC1L1	SEQ ID NO: 13
Back-translated polynucleotide sequence of human NPC1L1	SEQ ID NO: 51

A human NPC1L1 is also disclosed under Genbank Accession Number AF192522. As discussed below, the nucleotide sequence of the rat *NPC1L1* set forth in SEQ ID NO: 1 was obtained from an expressed sequence tag (EST) from a rat jejunum enterocyte cDNA library. SEQ ID NOs: 5-7 include partial nucleotide

sequences of three independent cDNA clones. The downstream sequence of the SEQ ID NO: 5 EST (603662080F1) were determined; the sequencing data from these experiments are set forth in SEQ ID NO: 8. The upstream sequences were also determined; these data are set forth in SEQ ID NO: 9.

SEQ ID NOs: 43 and 44 are the nucleotide and amino acid sequence, respectively, of human NPC1L1 which is disclosed under Genbank Accession No.: AF192522 (see Davies, *et al.*, (2000) Genomics 65(2): 137-45).

SEQ ID NO: 45 is the nucleotide sequence of a mouse *NPC1L1* which is disclosed under Genbank Accession No. AK078947.

NPC1L1 mediates intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption. Inhibition of NPC1L1 in a patient is a useful method for reducing intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption and serum sterol (*e.g.*, cholesterol) or 5α-stanol in the patient. Reducing the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption and serum sterol (*e.g.*, cholesterol) or 5α-stanol in a patient is a useful way in which to treat or prevent the occurrence of atherosclerosis, particularly diet-induced atherosclerosis.

As used herein, the term "sterol" includes, but is not limited to, cholesterol and phytosterols (including, but not limited to, sitosterol, campesterol, stigmasterol and avenosterol).

As used herein, the term "5α-stanol" includes, but is not limited to, cholestanol, 5α-campestanol and 5α-sitostanol.

Without being limited by the present hypothesis, the examples present a better understanding of the putative molecular interaction between NPC1L1 and cholesterol. In this regard, one of the more interesting features of NPC1L1 is that it contains the sterol-sensing domain (SSD) originally observed in SCAP (SREBP cleavage-activating protein). SCAP controls activation of sterol regulatory element binding proteins (SREBP), a transcription factor which controls more than 35 genes related to lipid and cholesterol homeostasis (Brown, M.S. & Goldstein, J.L. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc. Natl. Acad. Sci. U.S.A.* 96, 11041-11048 (1999)). The SSD, consisting of ~180 amino acids in a packet of 5 putative membrane-spanning helices, also serves a regulatory function in two key enzymes on the cholesterol biosynthesis pathway and is present in the receptor Patched. Recently, high affinity binding of cholesterol to the

SSD on SCAP has been demonstrated (Radhakrishnan, A., Sun, L., Kwon, H.J., Brown, M.S. & Goldstein, J.L., "Direct binding of cholesterol to the purified membrane region of SCAP: Mechanism for a sterol-sensing domain," *Mol. Cell* 15, 259-268 (2004)), suggesting that cholesterol may similarly bind to the SSD of NPC1L1, and raising the possibility that ezetimibe may compete with cholesterol for binding at this site.

Molecular Biology

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook, *et al.*, 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait, ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins, eds. (1985)); Transcription And Translation (B.D. Hames & S.J. Higgins, eds. (1984)); Animal Cell Culture (R.I. Freshney, ed. (1986)); Immobilized Cells And Enzymes (IRL Press, (1986)); B. Perbal, A Practical Guide To Molecular Cloning (1984); F.M. Ausubel, *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

The back-translated sequences of SEQ ID NO: 10 and of SEQ ID NO: 13 uses the single-letter code shown in Table 1 of Annex C, Appendix 2 of the PCT Administrative Instruction in the Manual of Patent Examination Procedure.

A "polynucleotide", "nucleic acid " or "nucleic acid molecule" may refer to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in single stranded form, double-stranded form or otherwise.

A "polynucleotide sequence", "nucleic acid sequence" or "nucleotide sequence" is a series of nucleotide bases (also called "nucleotides") in a nucleic acid, such as DNA or RNA, and means any chain of two or more nucleotides.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in production of the product.

The term "gene" means a DNA sequence that codes for or corresponds to a particular sequence of ribonucleotides or amino acids which comprise all or part of one or more RNA molecules, proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine, for example, the conditions under which the gene is expressed. Genes may be transcribed from DNA to RNA which may or may not be translated into an amino acid sequence.

The present invention includes nucleic acid fragments of any of SEQ ID NOs: 1, 5-11 or 13. A nucleic acid "fragment" includes at least about 30 (*e.g.*, 31, 32, 33, 34), preferably at least about 35 (e.g, 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34), more preferably at least about 45 (*e.g.*, 35, 36, 37, 38, 39, 40, 41, 42, 43 or 44), and most preferably at least about 126 or more contiguous nucleotides (*e.g.*, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 1000 or 1200) from any of SEQ ID NOs: 1, 5-11 or 13.

The present invention also includes nucleic acid fragments consisting of at least about 7 (*e.g.*, 9, 12, 17, 19), preferably at least about 20 (*e.g.*, 30, 40, 50, 60), more preferably about 70 (*e.g.*, 80, 90, 95), yet more preferably at least about 100 (*e.g.*, 105, 110, 114) and even more preferably at least about 115 (*e.g.*, 117, 119, 120, 122, 124, 125, 126) contiguous nucleotides from any of SEQ ID NOs: 1, 5-11 or 13.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of no more than about 100 nucleotides (*e.g.*, 30, 40, 50, 60, 70, 80, or 90), that may be hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest. Oligonucleotides can be labeled, *e.g.*, by incorporation of ^{32}P-nucleotides, ^{3}H-nucleotides, ^{14}C-nucleotides, ^{35}S-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of the gene, or to detect the presence of nucleic acids. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer.

A "protein sequence", "peptide sequence" or "polypeptide sequence" or "amino acid sequence" may refer to a series of two or more amino acids in a protein, peptide or polypeptide.

"Protein", "peptide" or "polypeptide" includes a contiguous string of two or more amino acids. Preferred peptides of the invention include those set forth in any of SEQ ID NOs: 2 or 12 as well as variants and fragments thereof. Such fragments preferably comprise at least about 10 (*e.g.*, 11, 12, 13, 14, 15, 16, 17, 18 or 19), more preferably at least about 20 (*e.g.*, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40), and yet more preferably at least about 42 (*e.g.*, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 110, 120 or 130) or more contiguous amino acid residues from any of SEQ ID NOs: 2 or 12.

The present invention also includes polypeptides, preferably antigenic polypeptides, consisting of at least about 7 (*e.g.*, 9, 10, 13, 15, 17, 19), preferably at least about 20 (*e.g.*, 22, 24, 26, 28), yet more preferably at least about 30 (*e.g.*, 32, 34, 36, 38) and even more preferably at least about 40 (*e.g.*, 41, 42) contiguous amino acids from any of SEQ ID NOs: 2 or 12.

The polypeptides of the invention can be produced by proteolytic cleavage of an intact peptide, by chemical synthesis or by the application of recombinant DNA technology and are not limited to polypeptides delineated by proteolytic cleavage sites. The polypeptides, either alone or cross-linked or conjugated to a carrier molecule to render them more immunogenic, are useful as antigens to elicit the production of antibodies and fragments thereof. The antibodies can be used, *e.g.*, in immunoassays for immunoaffinity purification or for inhibition of NPC1L1, etc.

The terms "isolated polynucleotide" or "isolated polypeptide" include a polynucleotide (*e.g.*, RNA or DNA molecule, or a mixed polymer) or a polypeptide, respectively, which are partially or fully separated from other components that are normally found in cells or in recombinant DNA expression systems. These components include, but are not limited to, cell membranes, cell walls, ribosomes, polymerases, serum components and extraneous genomic sequences.

An isolated polynucleotide or polypeptide will, preferably, be an essentially homogeneous composition of molecules but may contain some heterogeneity.

"Amplification" of DNA as used herein may denote the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. For a description of PCR see Saiki, *et al.*, Science (1988) 239: 487.

The term "host cell" includes any cell of any organism that is selected, modified, transfected, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example, the expression or replication, by the cell, of a gene, a DNA or RNA sequence or a protein. Preferred host cells include HEK-293 cells, chinese hamster ovary (CHO) cells, murine macrophage J774 cells or any other macrophage cell line and human intestinal epithelial Caco2 cells.

The nucleotide sequence of a nucleic acid may be determined by any method known in the art (*e.g.*, chemical sequencing or enzymatic sequencing). "Chemical sequencing" of DNA includes methods such as that of Maxam and Gilbert (1977) (Proc. Natl. Acad. Sci. USA 74: 560), in which DNA is randomly cleaved using individual base-specific reactions. "Enzymatic sequencing" of DNA includes methods such as that of Sanger (Sanger, *et al.*, (1977) Proc. Natl. Acad. Sci. USA 74: 5463).

The nucleic acids herein may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'- and 3'- non-coding regions, and the like.

In general, a "promoter" or "promoter sequence" is a DNA regulatory region capable of binding an RNA polymerase in a cell (*e.g.*, directly or through other promoter-bound proteins or substances) and initiating transcription of a coding sequence. A promoter sequence is, in general, bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at any level. Within the promoter sequence may be found a transcription initiation site (conveniently defined, for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. The promoter may be operably associated with other expression control sequences, including enhancer and repressor sequences or with a nucleic acid of the

invention. Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patent Nos. 5,385,839 and 5,168,062), the SV40 early promoter region (Benoist, *et al.*, (1981) Nature 290: 304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, (1980) Cell 22: 787-797), the herpes thymidine kinase promoter (Wagner, *et al.*, (1981) Proc. Natl. Acad. Sci. USA 78: 1441-1445), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, (1982) Nature 296: 39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Komaroff, *et al.*, (1978) Proc. Natl. Acad. Sci. USA 75: 3727-3731), or the *tac* promoter (DeBoer, *et al.*, (1983) Proc. Natl. Acad. Sci. USA 80: 21-25); see also "Useful proteins from recombinant bacteria" in Scientific American (1980) 242: 74-94; and promoter elements from yeast or other fungi such as the *Gal 4* promoter, the *ADC* (alcohol dehydrogenase) promoter, *PGK* (phosphoglycerol kinase) promoter or the alkaline phosphatase promoter.

A coding sequence is "under the control of", "functionally associated with" or "operably associated with" transcriptional and translational control sequences in a cell when the sequences direct RNA polymerase mediated transcription of the coding sequence into RNA, preferably mRNA, which then may be RNA spliced (if it contains introns) and, optionally, translated into a protein encoded by the coding sequence.

The terms "express" and "expression" mean allowing or causing the information in a gene, RNA or DNA sequence to become manifest; for example, producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene. A DNA sequence is expressed in or by a cell to form an "expression product" such as an RNA (*e.g.*, mRNA) or a protein. The expression product itself may also be said to be "expressed" by the cell.

The term "transformation" means the introduction of a nucleic acid into a cell. The introduced gene or sequence may be called a "clone". A host cell that receives the introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone." The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or from cells of a different genus or species.

The term "vector" includes a vehicle (*e.g.*, a plasmid) by which a DNA or RNA sequence can be introduced into a host cell, so as to transform the host and, optionally, promote expression and/or replication of the introduced sequence.

Vectors that can be used in this invention include plasmids, viruses, bacteriophage, integratable DNA fragments, and other vehicles that may facilitate introduction of the nucleic acids into the genome of the host. Plasmids are the most commonly used form of vector but all other forms of vectors which serve a similar function and which are, or become, known in the art are suitable for use herein. See, *e.g.*, Pouwels, *et al.*, Cloning Vectors: A Laboratory Manual, 1985 and Supplements, Elsevier, N.Y., and Rodriguez *et al.* (eds.), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, 1988, Buttersworth, Boston, MA.

The term "expression system" means a host cell and compatible vector which, under suitable conditions, can express a protein or nucleic acid which is carried by the vector and introduced to the host cell. Common expression systems include *E. coli* host cells and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors.

Expression of nucleic acids encoding the NPC1L1 polypeptides of this invention can be carried out by conventional methods in either prokaryotic or eukaryotic cells. Although *E. coli* host cells are employed most frequently in prokaryotic systems, many other bacteria, such as various strains of *Pseudomonas* and *Bacillus*, are known in the art and can be used as well. Suitable host cells for expressing nucleic acids encoding the NPC1L1 polypeptides include prokaryotes and higher eukaryotes. Prokaryotes include both gram-negative and gram-positive organisms, *e.g.*, *E. coli* and *B. subtilis*. Higher eukaryotes include established tissue culture cell lines from animal cells, both of non-mammalian origin, *e.g.*, insect cells, and birds, and of mammalian origin, *e.g.*, human, primates, and rodents.

Prokaryotic host-vector systems include a wide variety of vectors for many different species. A representative vector for amplifying DNA is pBR322 or many of its derivatives (*e.g.*, pUC18 or 19). Vectors that can be used to express the NPC1L1 polypeptides include, but are not limited to, those containing the *lac* promoter (pUC-series); *trp* promoter (pBR322-*trp*); *Ipp* promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as *ptac* (pDR540). See Brosius *et al.*, "Expression Vectors Employing Lambda-, *trp*-, lac-, and *Ipp*-derived

Promoters", in Rodriguez and Denhardt (eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, 1988, Buttersworth, Boston, pp. 205-236. Many polypeptides can be expressed, at high levels, in an *E.coli*/T7 expression system as disclosed in U.S. Patent Nos. 4,952,496; 5,693,489 and 5,869,320 and in Davanloo, P., *et al.*, (1984) Proc. Natl. Acad. Sci. USA 81: 2035-2039; Studier, F.W., *et al.*, (1986) J. Mol. Biol. 189: 113-130; Rosenberg, A. H., *et al.*, (1987) Gene 56: 125-135; and Dunn, J.J., *et al.*, (1988) Gene 68: 259.

Higher eukaryotic tissue culture cells may also be used for the recombinant production of the NPC1L1 polypeptides of the invention. Although any higher eukaryotic tissue culture cell line might be used, including insect baculovirus expression systems, mammalian cells are preferred. Transformation or transfection and propagation of such cells have become a routine procedure. Examples of useful cell lines include HeLa cells, chinese hamster ovary (CHO) cell lines, J774 cells, HEK-293 cells, Caco2 cells, baby rat kidney (BRK) cell lines, insect cell lines, bird cell lines, and monkey (COS) cell lines. Expression vectors for such cell lines usually include an origin of replication, a promoter, a translation initiation site, RNA splice sites (if genomic DNA is used), a polyadenylation site, and a transcription termination site. These vectors also, usually, contain a selection gene or amplification gene. Suitable expression vectors may be plasmids, viruses, or retroviruses carrying promoters derived, *e.g.*, from such sources as adenovirus, SV40, parvoviruses, vaccinia virus, or cytomegalovirus. Examples of expression vectors include pCR®3.1, pCDNA1, pCD (Okayama, *et al.*, (1985) Mol. Cell Biol. 5: 1136), pMC1neo Poly-A (Thomas, *et al.*, (1987) Cell 51: 503), pREP8, pSVSPORT and derivatives thereof, and baculovirus vectors such as pAC373 or pAC610. One embodiment of the invention includes membrane bound NPC1L1. In this embodiment, NPC1L1 can be expressed in the cell membrane of a eukaryotic cell and the membrane bound protein can be isolated from the cell by conventional methods which are known in the art.

The present invention also includes fusions which include the NPC1L1 polypeptides and *NPC1L1* polynucleotides of the present invention and a second polypeptide or polynucleotide moiety, which may be referred to as a "tag". The fusions of the present invention may comprise any of the polynucleotides or polypeptides set forth in Table 1 or any subsequence or fragment thereof (discussed

above). The fused polypeptides of the invention may be conveniently constructed, for example, by insertion of a polynucleotide of the invention or fragment thereof into an expression vector. The fusions of the invention may include tags which facilitate purification or detection. Such tags include glutathione-S-transferase (GST), hexahistidine (His6) tags, maltose binding protein (MBP) tags, haemagglutinin (HA) tags, cellulose binding protein (CBP) tags and myc tags. Detectable tags such as ^{32}P, ^{35}S, ^{3}H, ^{99m}Tc, ^{123}I, ^{111}In, ^{68}Ga, ^{18}F, ^{125}I, ^{131}I, ^{113m}In, ^{76}Br, ^{67}Ga, ^{99m}Tc, ^{123}I, ^{111}In and ^{68}Ga may also be used to label the polypeptides and polynucleotides of the invention. Methods for constructing and using such fusions are very conventional and well known in the art.

Modifications (*e.g.*, post-translational modifications) that occur in a polypeptide often will be a function of how it is made. For polypeptides made by expressing a cloned gene in a host, for instance, the nature and extent of the modifications, in large part, will be determined by the host cell's post-translational modification capacity and the modification signals present in the polypeptide amino acid sequence. For instance, as is well known, glycosylation often does not occur in bacterial hosts such as *E. coli*. Accordingly, when glycosylation is desired, a polypeptide can be expressed in a glycosylating host, generally a eukaryotic cell. Insect cells often carry out post-translational glycosylations which are similar to those of mammalian cells. For this reason, insect cell expression systems have been developed to express, efficiently, mammalian proteins having native patterns of glycosylation. An insect cell which may be used in this invention is any cell derived from an organism of the class *Insecta*. Preferably, the insect is *Spodoptera fruigiperda* (Sf9 or Sf21) or *Trichoplusia ni* (High 5). Examples of insect expression systems that can be used with the present invention, for example to produce NPC1L1 polypeptide, include Bac-To-Bac (Invitrogen Corporation, Carlsbad, CA) or Gateway (Invitrogen Corporation, Carlsbad, CA). If desired, deglycosylation enzymes can be used to remove carbohydrates attached during production in eukaryotic expression systems.

Other modifications may also include addition of aliphatic esters or amides to the polypeptide carboxyl terminus. The present invention also includes analogs of the NPC1L1 polypeptides which contain modifications, such as incorporation of unnatural amino acid residues, or phosphorylated amino acid

residues such as phosphotyrosine, phosphoserine or phosphothreonine residues. Other potential modifications include sulfonation, biotinylation, or the addition of other moieties. For example, the NPC1L1 polypeptides of the invention may be appended with a polymer which increases the half-life of the peptide in the body of a subject. Preferred polymers include polyethylene glycol (PEG) (*e.g.*, PEG with a molecular weight of 2 kDa, 5 kDa, 10 kDa, 12 kDa, 20 kDa, 30 kDa and 40 kDa), dextran and monomethoxypolyethylene glycol (mPEG).

The peptides of the invention may also be cyclized. Specifically, the amino- and carboxy-terminal residues of an NPC1L1 polypeptide or two internal residues of an NPC1L1 polypeptide of the invention can be fused to create a cyclized peptide. Methods for cyclizing peptides are conventional and very well known in the art; for example, see Gurrath, *et al.*, (1992) Eur. J. Biochem. 210: 911-921.

The present invention contemplates any superficial or slight modification to the amino acid or nucleotide sequences which correspond to the polypeptides of the invention. In particular, the present invention contemplates sequence conservative variants of the nucleic acids which encode the polypeptides of the invention. "Sequence-conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon results in no alteration in the amino acid encoded at that position. Function-conservative variants of the polypeptides of the invention are also contemplated by the present invention. "Function-conservative variants" are those in which one or more amino acid residues in a protein or enzyme have been changed without altering the overall conformation and function of the polypeptide, including, but, by no means, limited to, replacement of an amino acid with one having similar properties. Amino acids with similar properties are well known in the art. For example, polar/hydrophilic amino acids which may be interchangeable include asparagine, glutamine, serine, cysteine, threonine, lysine, arginine, histidine, aspartic acid and glutamic acid; nonpolar/hydrophobic amino acids which may be interchangeable include glycine, alanine, valine, leucine, isoleucine, proline, tyrosine, phenylalanine, tryptophan and methionine; acidic amino acids, which may be interchangeable include aspartic acid and glutamic acid and basic amino acids, which may be interchangeable include histidine, lysine and arginine.

The present invention includes polynucleotides encoding rat, human or mouse NPC1L1 and fragments thereof as well as nucleic acids which hybridize to the polynucleotides. Preferably, the nucleic acids hybridize under low stringency conditions, more preferably under moderate stringency conditions and most preferably under high stringency conditions. A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook, *et al.*, supra). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Typical low stringency hybridization conditions are 55°C, 5X SSC, 0.1% SDS, 0.25% milk, and no formamide at 42°C; or 30% formamide, 5X SSC, 0.5% SDS at 42°C. Typical, moderate stringency hybridization conditions are similar to the low stringency conditions except the hybridization is carried out in 40% formamide, with 5X or 6X SSC at 42°C. High stringency hybridization conditions are similar to low stringency conditions except the hybridization conditions are carried out in 50% formamide, 5X or 6X SSC and, optionally, at a higher temperature (*e.g.*, higher than 42°C: 57°C, 59°C, 60°C, 62°C, 63°C, 65°C or 68°C). In general, SSC is 0.15M NaC1 and 0.015M Na-citrate. Hybridization requires that the two nucleic acids contain complementary sequences, although, depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the higher the stringency under which the nucleic acids may hybridize. For hybrids of greater than 100 nucleotides in length, equations for calculating the melting temperature have been derived (see Sambrook, *et al.*, supra, 9.50-9.51). For hybridization with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook, *et al.*, supra).

Also included in the present invention are polynucleotides comprising nucleotide sequences and polypeptides comprising amino acid sequences which are at least about 70% identical, preferably at least about 80% identical, more preferably at least about 90% identical and most preferably at least about 95% identical (*e.g.*, 95%,

96%, 97%, 98%, 99%, 100%) to the reference rat *NPC1L1* nucleotide (*e.g.*, any of SEQ ID NOs: 1 or 5-10) and amino acid sequences (*e.g.*, SEQ ID NO: 2), reference human *NPC1L1* nucleotide (*e.g.*, SEQ ID NO: 3) and amino acid sequences (*e.g.*, SEQ ID NO: 4) or the reference mouse *NPC1L1* nucleotide (*e.g.*, any of SEQ ID NOs: 11 or 13) and amino acid sequences (*e.g.*, SEQ ID NO: 12), when the comparison is performed by a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences. Polypeptides comprising amino acid sequences which are at least about 70% similar, preferably at least about 80% similar, more preferably at least about 90% similar and most preferably at least about 95% similar (*e.g.*, 95%, 96%, 97%, 98%, 99%, 100%) to the reference rat NPC1L1 amino acid sequence of SEQ ID NO: 2, reference human NPC1L1 amino acid sequence of SEQ ID NO: 4 or the reference mouse NPC1L1 amino acid sequence of SEQ ID NO: 12, when the comparison is performed with a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences, are also included in the present invention.

Sequence identity refers to exact matches between the nucleotides or amino acids of two sequences which are being compared. Sequence similarity refers to both exact matches between the amino acids of two polypeptides which are being compared in addition to matches between nonidentical, biochemically related amino acids. Biochemically related amino acids which share similar properties and may be interchangeable are discussed above.

The following references regarding the BLAST algorithm are herein incorporated by reference: **BLAST ALGORITHMS**: Altschul, S.F., *et al.*, (1990) J. Mol. Biol. 215: 403-410; Gish, W., *et al.*, (1993) Nature Genet. 3: 266-272; Madden, T.L., *et al.*, (1996) Meth. Enzymol. 266: 131-141; Altschul, S.F., *et al.*, (1997) Nucleic Acids Res. 25: 3389-3402; Zhang, J., *et al.*, (1997) Genome Res. 7: 649-656; Wootton, J.C., *et al.*, (1993) Comput. Chem. 17: 149-163; Hancock, J.M., *et al.*, (1994) Comput. Appl. Biosci. 10: 67-70; **ALIGNMENT SCORING SYSTEMS**: Dayhoff, M.O., *et al.*, "A model of evolutionary change in proteins" in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 345-352, Natl. Biomed. Res. Found., Washington, DC; Schwartz, R.M., *et al.*, "Matrices

for detecting distant relationships" in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 353-358, Natl. Biomed. Res. Found., Washington, DC; Altschul, S.F., (1991) J. Mol. Biol. 219: 555-565; States, D.J., *et al.*, (1991) Methods 3: 66-70; Henikoff, S., *et al.*, (1992) Proc. Natl. Acad. Sci. USA 89: 10915-10919; Altschul, S.F., *et al.*, (1993) J. Mol. Evol. 36: 290-300; **ALIGNMENT STATISTICS**: Karlin, S., *et al.*, (1990) Proc. Natl. Acad. Sci. USA 87: 2264-2268; Karlin, S., *et al.*, (1993) Proc. Natl. Acad. Sci. USA 90: 5873-5877; Dembo, A., *et al.*, (1994) Ann. Prob. 22: 2022-2039; and Altschul, S.F. "Evaluating the statistical significance of multiple distinct local alignments" in Theoretical and Computational Methods in Genome Research (S. Suhai, ed.), (1997) pp. 1-14, Plenum, New York.

Protein Purification

The proteins, polypeptides and antigenic fragments of this invention can be purified by standard methods, including, but not limited to, salt or alcohol precipitation, affinity chromatography (*e.g.*, used in conjunction with a purification tagged NPC1L1 polypeptide as discussed above), preparative disc-gel electrophoresis, isoelectric focusing, high pressure liquid chromatography (HPLC), reversed-phase HPLC, gel filtration, cation and anion exchange and partition chromatography, and countercurrent distribution. Such purification methods are well known in the art and are disclosed, *e.g.*, in "Guide to Protein Purification", Methods in Enzymology, Vol. 182, M. Deutscher, Ed., 1990, Academic Press, New York, NY.

Purification steps can be followed by performance of assays for receptor binding activity as described below. Particularly where an NPC1L1 polypeptide is being isolated from a cellular or tissue source, it is preferable to include one or more inhibitors of proteolytic enzymes in the assay system, such as phenylmethanesulfonyl fluoride (PMSF), Pefabloc SC, pepstatin, leupeptin, chymostatin and EDTA.

Antibody Molecules

Antigenic (including immunogenic) fragments of the NPC1L1 polypeptides of the invention are within the scope of the present invention (*e.g.*, 42 or more contiguous amino acids from SEQ ID NO: 2, 4 or 12). The antigenic peptides may be useful, *inter alia*, for preparing isolated antibody molecules which recognize NPC1L1. Isolated anti-NPC1L1 antibody molecules are useful NPC1L1 ligands.

An antigen is any molecule that can bind specifically to an antibody. Some antigens cannot, by themselves, elicit antibody production. Those that can induce antibody production are immunogens.

Preferably, isolated anti-NPC1L1 antibodies recognize an antigenic peptide comprising an amino acid sequence selected from SEQ ID NOs: 39-42 (*e.g.*, an antigen derived from rat NPC1L1). More preferably, the antibody is A0715, A0716, A0717, A0718, A0867, A0868, A1801 or A1802.

The term "antibody molecule " includes, but is not limited to, antibodies and fragments (preferably antigen-binding fragments) thereof. The term includes monoclonal antibodies, polyclonal antibodies, bispecific antibodies, Fab antibody fragments, $F(ab)_2$ antibody fragments, Fv antibody fragments (*e.g.*, V_H or V_L), single chain Fv antibody fragments and dsFv antibody fragments. Furthermore, the antibody molecules of the invention may be fully human antibodies, mouse antibodies, rat antibodies, rabbit antibodies, goat antibodies, chicken antibodies, humanized antibodies or chimeric antibodies.

Although it is not always necessary, when NPC1L1 polypeptides are used as antigens to elicit antibody production in an immunologically competent host, smaller antigenic fragments are, preferably, first rendered more immunogenic by cross-linking or concatenation, or by coupling to an immunogenic carrier molecule (i.e., a macromolecule having the property of independently eliciting an immunological response in a host animal, such as diptheria toxin or tetanus). Cross-linking or conjugation to a carrier molecule may be required because small polypeptide fragments sometimes act as haptens (molecules which are capable of specifically binding to an antibody but incapable of eliciting antibody production, i.e., they are not immunogenic). Conjugation of such fragments to an immunogenic carrier molecule renders them more immunogenic through what is commonly known as the "carrier effect".

Carrier molecules include, *e.g.*, proteins and natural or synthetic polymeric compounds such as polypeptides, polysaccharides, lipopolysaccharides, etc. Protein carrier molecules are especially preferred, including, but not limited to, keyhole limpet hemocyanin and mammalian serum proteins such as human or bovine gammaglobulin, human, bovine or rabbit serum albumin, or methylated or other derivatives of such proteins. Other protein carriers will be apparent to those skilled in

the art. Preferably, the protein carrier will be foreign to the host animal in which antibodies against the fragments are to be elicited.

Covalent coupling to the carrier molecule can be achieved using methods well known in the art, the exact choice of which will be dictated by the nature of the carrier molecule used. When the immunogenic carrier molecule is a protein, the fragments of the invention can be coupled, *e.g.*, using water-soluble carbodiimides such as dicyclohexylcarbodiimide or glutaraldehyde.

Coupling agents, such as these, can also be used to cross-link the fragments to themselves without the use of a separate carrier molecule. Such cross-linking into aggregates can also increase immunogenicity. Immunogenicity can also be increased by the use of known adjuvants, alone or in combination with coupling or aggregation.

Adjuvants for the vaccination of animals include, but are not limited to, Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate); Freund's complete or incomplete adjuvant; mineral gels such as aluminum hydroxide, aluminum phosphate and alum; surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dioctadecyl-N',N'-bis(2-hydroxymethyl) propanediamine, methoxyhexadecylglycerol and pluronic polyols; polyanions such as pyran, dextran sulfate, poly IC, polyacrylic acid and carbopol; peptides such as muramyl dipeptide, dimethylglycine and tuftsin; and oil emulsions. The polypeptides could also be administered following incorporation into liposomes or other microcarriers.

Information concerning adjuvants and various aspects of immunoassays are disclosed, *e.g.*, in the series by P. Tijssen, Practice and Theory of Enzyme Immunoassays, 3rd Edition, 1987, Elsevier, New York. Other useful references covering methods for preparing polyclonal antisera include Microbiology, 1969, Hoeber Medical Division, Harper and Row; Landsteiner, Specificity of Serological Reactions, 1962, Dover Publications, New York, and Williams, *et al.*, Methods in Immunology and Immunochemistry, Vol. 1, 1967, Academic Press, New York.

The anti-NPC1L1 antibody molecules of the invention preferably recognize human, mouse or rat NPC1L1; however, the present invention includes antibody molecules which recognize NPC1L1 from any species, preferably mammals

(*e.g.*, cat, sheep or horse). The present invention also includes complexes comprising an NPC1L1 polypeptide of the invention and an anti-NPC1L1 antibody molecule. Such complexes can be made by simply contacting the antibody molecule with its cognate polypeptide.

Various methods may be used to make the antibody molecules of the invention. Human antibodies can be made, for example, by methods which are similar to those disclosed in U.S. Patent Nos. 5,625,126; 5,877,397; 6,255,458; 6,023,010 and 5,874,299.

Hybridoma cells which produce the monoclonal anti-NPC1L1 antibodies may be produced by methods which are commonly known in the art. These methods include, but are not limited to, the hybridoma technique originally developed by Kohler, *et al.*, (1975) (Nature 256: 495-497), as well as the trioma technique (Hering, *et al.*, (1988) Biomed. Biochim. Acta. 47: 211-216 and Hagiwara, *et al.*, (1993) Hum. Antibod. Hybridomas 4: 15), the human B-cell hybridoma technique (Kozbor, *et al.*, (1983) Immunology Today 4: 72 and Cote, *et al.*, (1983) Proc. Natl. Acad. Sci. U.S.A 80: 2026-2030), and the EBV-hybridoma technique (Cole, *et al.*, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1985). ELISA may be used to determine if hybridoma cells are expressing anti-NPC1L1 antibodies.

The anti-NPC1L1 antibody molecules of the present invention may also be produced recombinantly (*e.g.*, in an *E.coli*/T7 expression system as discussed above). In this embodiment, nucleic acids encoding the antibody molecules of the invention (*e.g.*, V_H or V_L) may be inserted into a pet-based plasmid and expressed in the *E.coli*/T7 system. There are several methods by which to produce recombinant antibodies which are known in the art. An example of a method for recombinant production of antibodies is disclosed in U.S. Patent No. 4,816,567. See also Skerra, A., *et al.*, (1988) Science 240: 1038-1041; Better, M., *et al.*, (1988) Science 240: 1041-1043 and Bird, R.E., *et al.*, (1988) Science 242: 423-426.

The term "monoclonal antibody," includes an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible, naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Monoclonal antibodies are

advantageous in that they may be synthesized by a hybridoma culture, essentially uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. The monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method as described by Kohler, *et al.*, (1975) Nature 256: 495.

The term "polyclonal antibody" includes an antibody which was produced among or in the presence of one or more other, non-identical antibodies. In general, polyclonal antibodies are produced from a B-lymphocyte in the presence of several other B-lymphocytes which produced non-identical antibodies. Typically, polyclonal antibodies are obtained directly from an immunized animal (*e.g.*, a rabbit).

A "bispecific antibody" comprises two different antigen binding regions which bind to distinct antigens. Bispecific antibodies, as well as methods of making and using the antibodies, are conventional and very well known in the art.

Anti-idiotypic antibodies or anti-idiotypes are antibodies directed against the antigen-combining region or variable region (called the idiotype) of another antibody molecule. As disclosed by Jerne (Jerne, N. K., (1974) Ann. Immunol. (Paris) 125c: 373 and Jerne, N. K., *et al.*, (1982) EMBO 1: 234), immunization with an antibody molecule expressing a paratope (antigen-combining site) for a given antigen (*e.g.*, NPC1L1) will produce a group of anti-antibodies, some of which share, with the antigen, a complementary structure to the paratope. Immunization with a subpopulation of the anti-idiotypic antibodies will, in turn, produce a subpopulation of antibodies or immune cell subsets that are reactive to the initial antigen.

The term "fully human antibody" refers to an antibody which comprises human immunoglobulin sequences only. Similarly, "mouse antibody" refers to an antibody which comprises mouse immunoglobulin sequences only.

"Human/mouse chimeric antibody" refers to an antibody which comprises a mouse variable region (V_H and V_L) fused to a human constant region.

"Humanized" anti-NPC1L1 antibodies are also within the scope of the present invention. Humanized forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, which contain minimal sequence derived from non-

human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region of the recipient are replaced by residues from a complementary determining region of a nonhuman species (donor antibody), such as mouse, rat or rabbit, having a desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are also replaced by corresponding non-human residues.

"Single-chain Fv" or "sFv" antibody fragments include the V_H and/or V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the sFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. Techniques described for the production of single chain antibodies (U.S. Patent Nos. 5,476,786; 5,132,405 and 4,946,778) can be adapted to produce anti-NPC1L1 specific, single chain antibodies. For a review of sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore, eds., Springer-Verlag, N.Y., pp. 269-315 (1994).

"Disulfide stabilized Fv fragments" and "dsFv" include molecules having a variable heavy chain (V_H) and/or a variable light chain (V_L) which are linked by a disulfide bridge.

Antibody fragments within the scope of the present invention also include $F(ab)_2$ fragments which may be produced by enzymatic cleavage of an IgG by, for example, pepsin. Fab fragments may be produced by, for example, reduction of $F(ab)_2$ with dithiothreitol or mercaptoethylamine.

An FV fragment is a V_L or V_H region.

Depending on the amino acid sequences of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are at least five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG-1, IgG-2, IgG-3 and IgG-4; IgA-1 and IgA-2.

The anti-NPC1L1 antibody molecules of the invention may also be conjugated to a chemical moiety. The chemical moiety may be, *inter alia,* a polymer, a radionuclide or a cytotoxic factor. Preferably, the chemical moiety is a polymer which increases the half-life of the antibody molecule in the body of a subject.

Suitable polymers include, but are by no means limited to, polyethylene glycol (PEG) (*e.g.*, PEG with a molecular weight of 2kDa, 5kDa, 10kDa, 12kDa, 20kDa, 30kDa or 40kDa), dextran and monomethoxypolyethylene glycol (mPEG). Methods for producing PEGylated anti-IL8 antibodies which are described in U.S. Patent No. 6,133,426 can be applied to the production of PEGylated anti-NPC1L1 antibodies of the invention. Lee, *et al.*, (1999) (Bioconj. Chem. 10: 973-981) discloses PEG conjugated single-chain antibodies. Wen, *et al.*, (2001) (Bioconj. Chem. 12: 545-553) discloses conjugating antibodies with PEG which is attached to a radiometal chelator (diethylenetriaminpentaacetic acid (DTPA)).

The antibody molecules of the invention may also be conjugated with labels such as ^{99}Tc, ^{90}Y, ^{111}In, ^{32}P, ^{14}C, ^{125}I, ^{3}H, ^{131}I, ^{11}C, ^{15}O, ^{13}N, ^{18}F, ^{35}S, ^{51}Cr, ^{57}To, ^{226}Ra, ^{60}Co, ^{59}Fe, ^{57}Se, ^{152}Eu, ^{67}CU, ^{217}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{234}Th, ^{40}K, ^{157}Gd, ^{55}Mn, ^{52}Tr or ^{56}Fe.

The antibody molecules of the invention may also be conjugated with fluorescent or chemilluminescent labels, including fluorophores such as rare earth chelates, fluorescein and its derivatives, rhodamine and its derivatives, isothiocyanate, phycoerythrin, phycocyanin, allophycocyanin, o-phthaladehyde, fluorescamine, ^{152}Eu, dansyl, umbelliferone, luciferin, luminal label, isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridimium salt label, an oxalate ester label, an aequorin label, 2,3-dihydrophthalazinediones, biotin/avidin, spin labels and stable free radicals.

The antibody molecules may also be conjugated to a cytotoxic factor such as diptheria toxin, *Pseudomonas aeruginosa* exotoxin A chain, ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins and compounds (*e.g.*, fatty acids), dianthin proteins, *Phytoiacca americana* proteins PAPI, PAPII, and PAP-S, *momordica charantia* inhibitor, curcin, crotin, *saponaria officinalis* inhibitor, mitogellin, restrictocin, phenomycin, and enomycin.

Any method known in the art for conjugating the antibody molecules of the invention to the various moieties may be employed, including those methods described by Hunter, *et al.*, (1962) Nature 144: 945; David, *et al.*, (1974) Biochemistry 13: 1014; Pain, *et al.*, (1981) J. Immunol. Meth. 40: 219; and Nygren, J., (1982) Histochem. and Cytochem. 30: 407.

Methods for conjugating antibodies are conventional and very well known in the art.

Screening Assays

The invention allows the identification of selective ligands of NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12) that may be useful in treatment and management of a variety of medical conditions, including elevated serum sterol (*e.g.*, cholesterol) or 5α-stanol. Thus, NPC1L1 of this invention can be employed in screening systems to identify ligands. These ligands may be agonists or antagonists of NPC1L1. Essentially, these assays provide methods for identifying ligands of NPC1L1 by using (1) NPC1L1, (2) an appropriate known NPC1L1 ligand, agonist or antagonist, for example, a sterol (such as cholesterol, phytosterols, including, but not limited to, sitosterol, campesterol, stigmasterol and avenosterol), a cholesterol oxidation product, a 5α-stanol (including, but not limited to, cholestanol, 5α-campestanol and 5α-sitostanol), a substituted azetidinone (*e.g.*, ezetimibe), BODIPY-ezetimibe (Altmann, *et al.*, (2002) Biochim. Biophys. Acta 1580(1): 77-93) or 4", 6"-bis[(2-fluorophenyl)carbamoyl]-beta-D-cellobiosyl derivative of 11-ketotigogenin as described in DeNinno, *et al.*, (1997) (J. Med. Chem. 40(16): 2547-54) or any substituted azetidinone, and (3) a sample to be tested for the presence of a candidate NPC1L1 ligand.

The term "specific" when used to describe binding of, for example, a ligand of NPC1L1 in a screening assay is a term of art which refers to the extent by which the ligand or antagonist (*e.g.*, substituted azetidinone, ezetimibe, sterol (such as cholesterol) or 5α-stanol) binds preferentially to NPC1L1 in comparison to other proteins in the assay system. For example, detection of the specific binding of a ligand of NPC1L1 binds specifically to NPC1L1 is made apparent when a signal generated in the assay to indicate such binding exceeds, to any extent, a signal generated in a negative control wherein, for example, NPC1L1 or ligand is absent. Furthermore, "specific binding" includes binding of a ligand either directly to NPC1L1 or indirectly, for example via another moiety, in a complex of which NPC1L1 is a part. The moiety to which an NPC1L1 ligand binds can be another protein or a post-translational modification of NPC1L1 (*e.g.*, a lipid chain or a carbohydrate chain).

Non-limiting examples of suitable substituted azetidinones for use in the screening assays include those disclosed in U.S. Patent Nos. RE37,721; 5,631,365; 5,767,115; 5,846,966; 5,688,990; 5,656,624; 5,624,920; 5,698,548; 5,756,470; 5,688,787; 5,306,817; 5,633,246; 5,627,176; 5,688,785; 5,744,467; 5,846,966; 5,728,827; 6,632,933 and U.S. Patent Application Publication No 2003/0105028-each of which is herein incorporated by reference in its entirety.

The present invention provides for a method by which to evaluate whether a sample contains an NPC1L1 ligand by determining whether the sample contains a candidate compound which competes for binding between the known ligand (*e.g.*, ezetimibe, ezetimibe-glucuronide, compound 2, etc.) and NPC1L1. The ligand may be an agonist or antagonist. In an embodiment of the invention, the binding of the known ligand (*e.g.*, ezetimibe, ezetimibe-glucuronide, compound **2**, etc.) to NPC1L1 is disrupted. The term "known ligand" refers to a compound which is known to bind to NPC1L1 and which can be detectably labeled for use in the screening assays and methods described herein. "Known ligands" include the substituted 2-azetidinone glucuronides which can be detectably labeled for use in screening assays as described herein.

Ezetimibe can be prepared by a variety of methods well know to those skilled in the art, for example such as are disclosed in U.S. Patents Nos. 5,631,365, 5,767,115, 5,846,966, 6,207,822, U.S. Patent Application Publication No. 2002/0193607 and PCT Patent Application WO 93/02048, each of which is incorporated herein by reference in its entirety.

"Sample", "candidate compound" or "candidate substance" refers to a compound or composition which is evaluated in a test or assay, for example, for the ability to bind to NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12) or a functional fragment thereof. The composition may comprise candidate compounds, such as small molecules, peptides, nucleotides, polynucleotides, subatomic particles (*e.g.*, α particles, β particles) or antibodies.

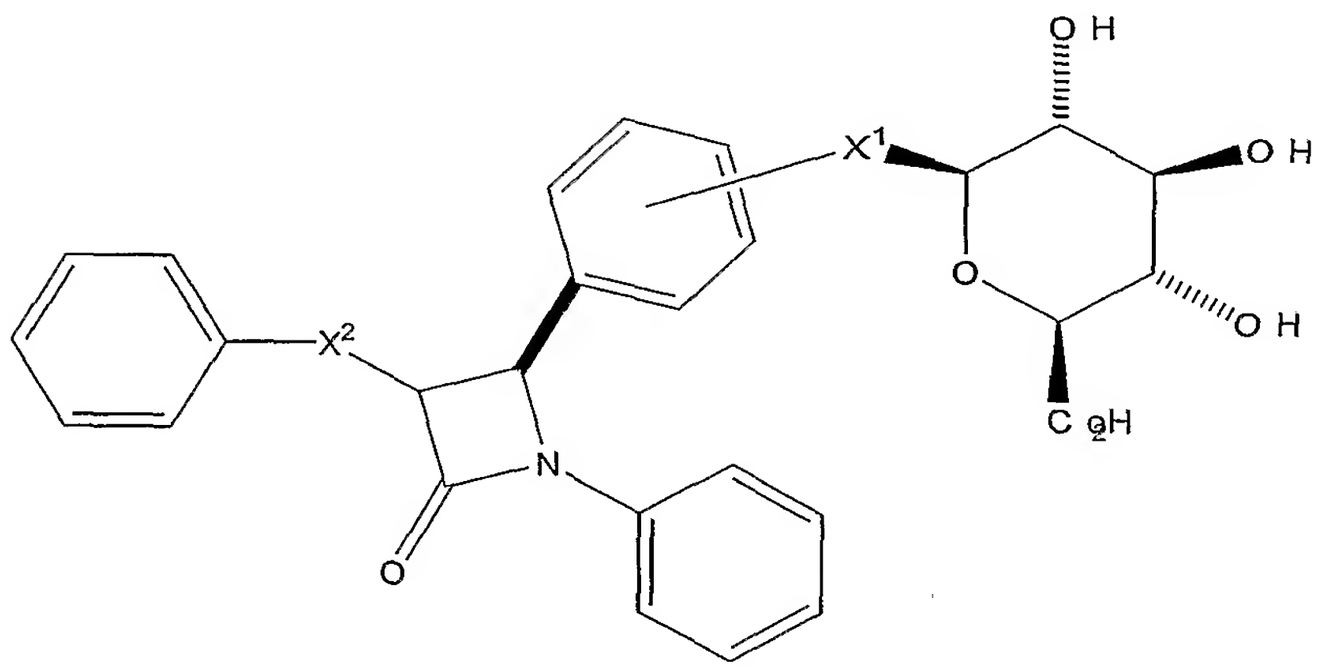
The present invention provides methods for identifying ligands of a compound that binds to NPC1L1 which involve contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and a candidate compound, and determining whether the candidate compound binds to NPC1L1, wherein binding of said candidate compound to NPC1L1 modulates

binding of the detectably labeled substituted 2-azetidinone to NPC1L1. The modulation of the binding of the substituted 2-azetidinone to NPC1L1 by the binding of the candidate compound to NPC1L1 indicates that the candidate compound is a ligand that binds to NPC1L1. It is also a good indication that the candidate compound may be an inhibitor of sterol and 5α-stanol absorption in vivo.

The present invention also provides a method for identifying a ligand of NPC1L1 comprising contacting NPC1L1 with a detectably labeled substituted 2-azetidinone, preferably substituted 2-azetidinone-glucuronide, and measuring the binding of NPC1L1 of the detectably labeled substituted 2-azetidinone in the presence and absence of a candidate compound, wherein decreased binding of the detectably labeled substituted 2-azetidinone to the NPC1L1 in the presence of the candidate compound indicates that said candidate compound is a ligand of NPC1L1 and is an inhibitor of sterol and 5α-stanol absorption.

The substituted 2-azetidinone is detectably labeled with ^{3}H, ^{35}S, ^{125}I, or a fluorescently labeled substituted 2-azetidinone. Preferably, the substituted 2-azetidinone is labeled with ^{35}S or ^{125}I, and particularly ^{35}S.

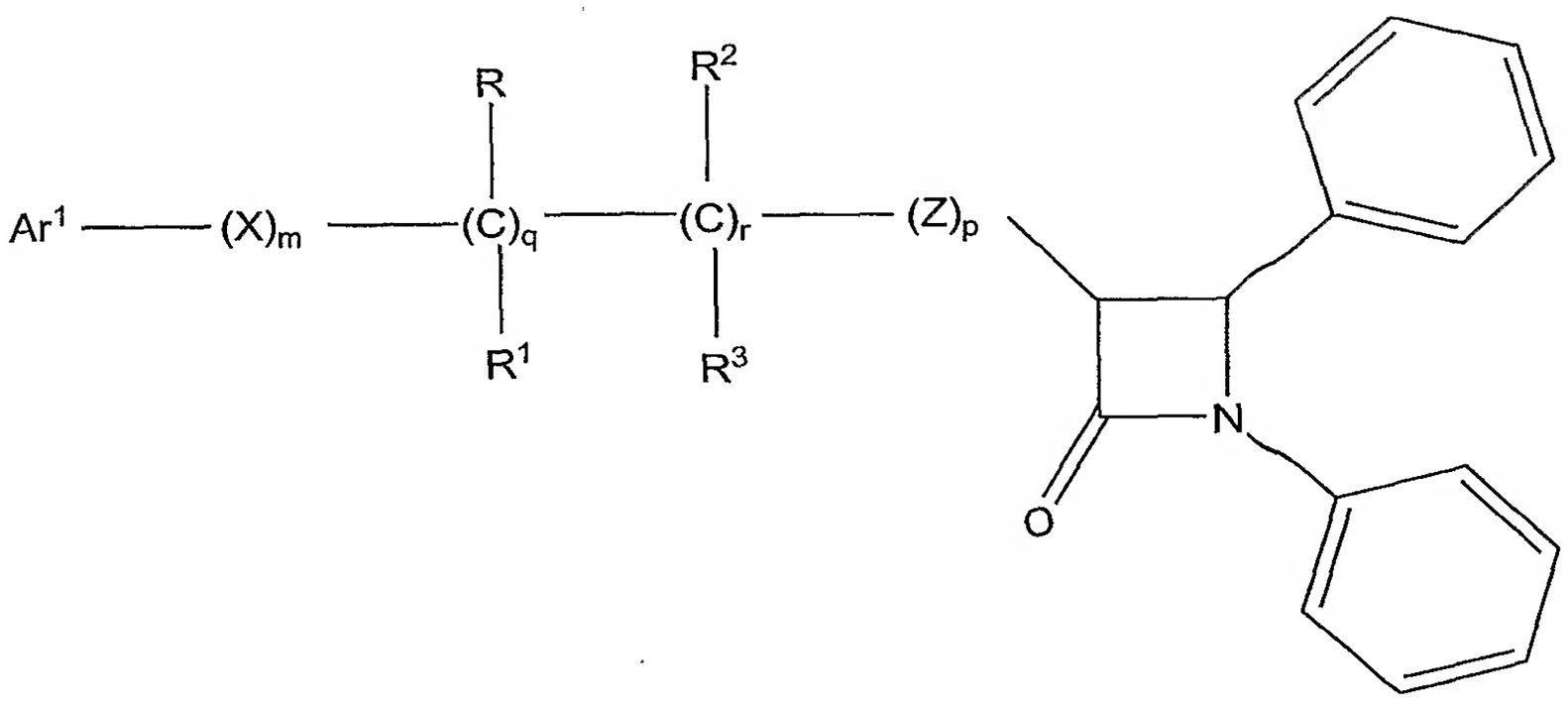
Preferably, the substituted 2-azetidinone is substituted 2-azetidinone-glucuronide. Compounds that are substituted 2-azetidinone-glucuronides are those having the following structure (I):



(I)

wherein X^1 represents a group that links the glucuronide to the 4-phenyl ring, for example but not limited to –O- or $-C_{1-3}$ alkyl-, X^2 represents an optionally substituted –alkanediyl-, and wherein any of the phenyl groups may be optionally substituted. Examples of the phenyl-X^2-moiety in structure (I) include

those represented at the 4-position on the 2-azetidinone structure shown below in structure (II). Additional examples of substituted 2-azetidinone-glucuronides include but are not limited to those described in U.S. Patent No. 5,756,470, WO02/066464 and US 2002/0137689. Additional examples of substituted 2-azetidinone-glucuronide compounds include those having the structure (II) and pharmaceutically acceptable salts and esters thereof as follows:



(II)

wherein:

Ar^1 is selected from the group consisting of aryl and R^4 -substituted aryl;

X, Y and Z are independently selected from the group consisting of $-CH_2-$, $-CH(C_{1-6}alkyl)-$ and $-C(C_{1-6}alkyl)_2-$;

R is selected from the group consisting of $-OR^6$, $-O(CO)R^6$, $-O(CO)OR^9$, $-O(CO)NR^6R^7$, a sugar residue, a disugar residue, a trisugar residue and a tetrasugar residue;

R^1 is selected from the group consisting of -H, $-C_{1-6}alkyl$ and aryl, or R and R^1 together are oxo;

R^2 is selected from the group consisting of $-OR^6$, $-O(CO)R^6$, $-O(CO)OR^9$ and $-O(CO)NR^6R^7$;

R^3 is selected from the group consisting of -H, $-C_{1-6}alkyl$ and aryl or R^2 and R^3 together are oxo;

q, r and t are each independently selected from 0 and 1;

m, n and p are each independently selected from 0, 1, 2, 3 and 4;

R^4 is 1-5 substituents independently selected at each occurrence from the group consisting of:

$-OR^5$, $-O(CO)R^5$, $-O(CO)OR^8$, $-O-C_{1-5}alkyl-OR^5$, $-O(CO)NR^5R^6$, $-NR^5R^6$, $-NR^5(CO)R^6$, $-NR^5(CO)OR^8$, $-NR^5(CO)NR^6R^7$, $-NR^5SO_2R^8$, $-COOR^5$, $-CONR^5R^6$, $-COR^5$,

$-SO_2NR^5R^6$, $-S(O)_tR^8$, $-O-C_{1-10}alkyl-COOR^5$, $-O-C_{1-10}alkyl-CONR^5R^6$ and fluoro;

R^5, R^6 and R^7 are independently selected at each occurrence from the group consisting of -H, C_{1-6}alkyl, aryl and aryl-substituted C_{1-6}alkyl;

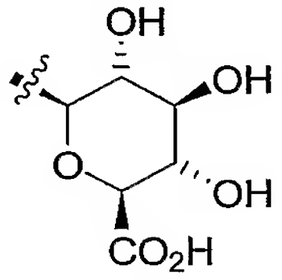
R^8 is independently selected from the group consisting of C_{1-6}alkyl, aryl and aryl-substituted C_{1-6}alkyl;

R^9 is selected from the group consisting of $-C\equiv C-CH_2-NR^{10}R^{11}$, $-C\equiv C-C(O)R^{13}$, and $-(CH_2)_3-NR^{10}R^{14}$;

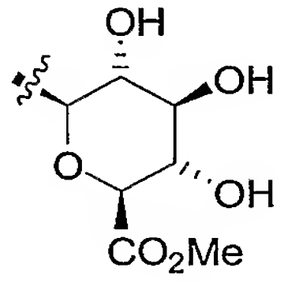
R^{10} is independently selected at each occurrence from –H and $-C_{1-3}$alkyl;

R^{11} is selected from the group consisting of –H, $-C_{1-3}$alkyl, $-C(O)-C_{1-3}$alkyl, $-C(O)-NR^{10}R^{10}$, $-SO_2-C_{1-3}$alkyl, and $-SO_2$-phenyl; and

R^{12} is selected from



and



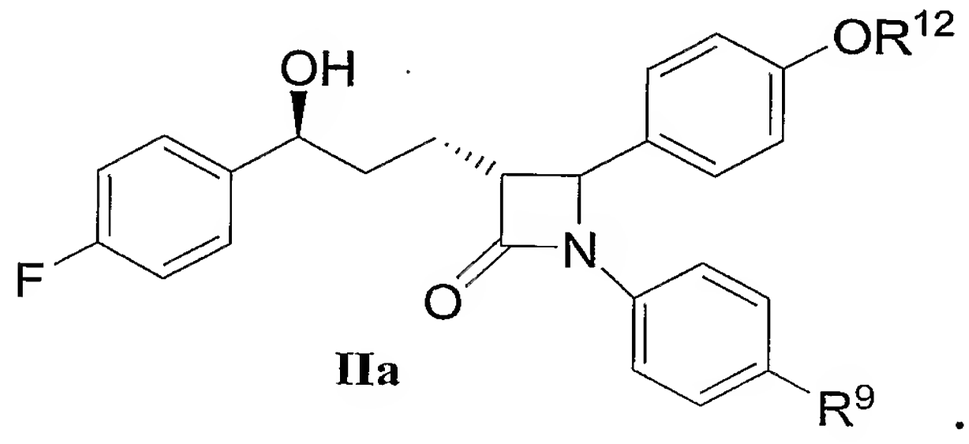
(referred to herein as "glucuronide") (referred to herein as "methyl ester glucuronide");

R^{13} is selected from the group consisting of –OH and $-NR^{10}R^{11}$; and

R^{14} is selected from the group consisting of $-C(O)-C_{1-3}$alkyl, $-C(O)-NR^{10}R^{10}$, $-SO_2-C_{1-3}$alkyl and $-SO_2$-phenyl.

In one embodiment of Formula II are compounds wherein q, r and t are each independently selected from 0 and 1; and m, n and p are each independently selected from 0, 1, 2, 3 and 4; provided that at least one of q and r is 1, and the sum of

m, n, p, q are r is 1, 2, 3, 4, 5 or 6; and provided that when p is 0 and r is 1, the sum of m, q and n is 1, 2, 3, 4, or 5. In a second embodiment of Formula II are compounds of Formula IIa,



IIa

In a class of each of these embodiments are compounds wherein R^9 is -C≡C–CH_2-$NR^{10}R^{11}$. In another class of each of these embodiments are compounds wherein R^9 contains an -SO_2- group, i.e., wherein R^9 is selected from the group consisting of _-C≡C-CH_2-$NR^{10}R^{11}$, -C≡C-C(O) $NR^{10}R^{11}$, –$(CH_2)_3$-NR^{10}-SO_2-C_{1-3}alkyl and -$(CH_2)_3$-NR^{10}-SO_2-phenyl, and R^{11} is selected from -SO_2-C_{1-3}alkyl, and –SO_2-phenyl.

The term "alkyl" is intended to include both branched- and straight-chain saturated aliphatic univalent hydrocarbon groups having the specified number of carbon atoms. Examples of alkyl groups include, but are not limited to, methyl (Me), ethyl (Et), n-propyl (Pr), n-butyl (Bu), n-pentyl, n-hexyl, and the isomers thereof such as isopropyl (i-Pr), isobutyl (i-Bu), secbutyl (s-Bu), tertbutyl (t-Bu), isopentyl, isohexyl and the like. If there is no specified prefix (such as "n-" for normal, "s-" for sec, "t-" for tert, "i-" for iso) with a named alkyl group, then it is intended that the named alkyl group is an n-alkyl group (i.e., "propyl" is "n-propyl"). The term "aryl" is intended to include phenyl (Ph), naphthyl, indenyl, tetrahydronaphthyl or indanyl. Phenyl is preferred.

Suitable protecting groups (designated as "PG" herein) for the hydroxyl groups of R^{12} when R^{12} is a glucuronide or methyl ester glucuronide include but are not limited to those that are known to be useful as carbohydrate protecting groups, such as for example benzyl, acetyl, benzoyl, *tert*-butyldiphenylsilyl, trimethylsilyl, *para*-methoxybenzyl, benzylidine, and methoxy methyl. Conditions required to selectively add and remove such protecting groups are

found in standard textbooks such as Greene, T, and Wuts, P. G. M., *Protective Groups in Organic Synthesis*, John Wiley & Sons, Inc., New York, NY, 1999.

Compounds of Formula II may contain one or more asymmetric centers and can thus occur as racemates and racemic mixtures, single enantiomers, enantiomeric mixtures, diastereomeric mixtures and individual diastereomers, and all such isomeric forms are within the scope of Formula II.

Radioactive isotopes of the compounds of Formula II are particularly useful in such assays, for example compounds of Formula II wherein sulfur is replaced with "hot" -^{35}S-, and particularly wherein the radioactive sulfur isotope is incorporated within the R^9 moiety. The use of all such radioactive isotopes of the compounds of Formula II in an assay for identifying NPC1L1 ligands is included within the scope of this invention.

The term "pharmaceutically acceptable salts" means non-toxic salts of the compounds of Formula II which are generally prepared by reacting the free acid with a suitable organic or inorganic base, particularly those formed from cations such as sodium, potassium, aluminum, calcium, lithium, magnesium, zinc and tetramethylammonium, as well as those salts formed from amines such as ammonia, ethylenediamine, N-methylglucamine, lysine, arginine, ornithine, choline, N,N'-dibenzylethylenediamine, chloroprocaine, diethanolamine, procaine, N-benzylphenethylamine, 1-p-chlorobenzyl-2-pyrrolidine-1'-yl-methylbenzimidazole, diethylamine, piperazine, morpholine, 2,4,4-trimethyl-2-pentamine and tris(hydroxymethyl)aminomethane.

When the compounds of Formula II are basic, salts may be prepared from pharmaceutically acceptable non-toxic acids, including inorganic and organic acids. Such acids include acetic, benzenesulfonic, benzoic, camphorsulfonic, citric, ethanesulfonic, fumaric, gluconic, glutamic, hydrobromic, hydrochloric, isethionic, lactic, maleic, malic, mandelic, methanesulfonic, mucic, nitric, pamoic, pantothenic, phosphoric, succinic, sulfuric, tartaric, p-toluenesulfonic acid, and the like. Particularly preferred are citric, hydrobromic, hydrochloric, maleic, phosphoric, sulfuric, and tartaric acids.

Examples of pharmaceutically acceptable esters include, but are not limited to, -C_{1-4} alkyl and –C_{1-4} alkyl substituted with phenyl, dimethylamino and acetylamino. "C_{1-4} alkyl" herein includes straight or branched aliphatic chains

containing from 1 to 4 carbon atoms, for example methyl, ethyl, n-propyl, n-butyl, iso-propyl, sec-butyl and tert-butyl.

The compounds of structural Formula II can be prepared according to the procedures of the following Scheme using appropriate materials, and are further exemplified by specific examples which follow. A variety of chromatographic techniques may be employed in the preparation of the compounds of Formula II. These techniques include, but are not limited to: High Performance Liquid Chromatography (including normal- reversed- and chiral-phase); Super Critical Fluid Chromatography; preparative Thin Layer Chromatography; flash chromatography with silica gel or reversed-phase silica gel; ion-exchange chromatography; and radial chromatography. All temperatures are degrees Celsius unless otherwise noted.

Some abbreviations used herein include:

Ac	Acyl ($CH_3C(O)$-)
Bn	benzyl
calc.	Calculated
Celite	Celite™ diatomaceous earth
Dess-Martin Periodinane	1,1,1-tris(acetyloxy)-1,1-dihydro-1,2-benzodoxol-3-(1*H*)-one
DMF	N,N-dimethylformamide
equiv.	Equivalent(s)
ES-MS	Electron Spray Ion-Mass Spectroscopy
EtOAc	Ethyl acetate
h	Hour(s)
HPLC	High performance liquid chromatography
min	Minute(s)
m.p.	Melting point
MS	Mass spectrum
r.t. (or rt)	Room temperature
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Tlc	Thin layer chromatography

The general Scheme below illustrates a method for the syntheses of compounds of structural formula II-4. All substituents are as defined in Formula II unless indicated otherwise. In this method, II-1 is treated with a terminal alkyne of

type II-2 in the presence of a suitable palladium catalyst such as tetrakistriphenylphosphine palladium(0) or [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II) or the like, and copper(I) iodide. The reaction is usually performed in an inert organic solvent such as DMF, between room temperature and 100 °C, for a period of 6-48 h, and the product is an internal alkyne of structural formula II-3. Alkyne II-2 may contain a radioactive atom such as 35S to provide the corresponding radiolabeled adduct upon reaction with II-1. Conversion of II-3 to II-4 can be achieved using a variety of hydrolytic methods known to those skilled in the art of organic synthesis. For example, a particularly mild hydrolysis protocol involves the treatment of II-3 with a tertiary amine base such as triethylamine, or diisopropylethylamine or the like, in a mixed solvent system comprising methanol and water. The product of the reaction is a compound of structural formula II-4. By utilizing the procedures described herein, one of ordinary skill in the art can readily prepare additional compounds of Formual II.

SCHEME

Two additional types of screening systems that can be used include a labeled-ligand binding assay (*e.g.*, direct binding assay or scintillation proximity assay (SPA)) and a "sterol (*e.g.*, cholesterol) or 5α-stanol uptake" assay. A labeled ligand, for use in the binding assay, can be obtained by labeling a sterol (*e.g.*, cholesterol) or a 5α-stanol or a known NPC1L1 agonist or antagonist with a measurable group (*e.g.*, ^{35}S, ^{125}I or ^{3}H). Various labeled forms of sterols (*e.g.*, cholesterol) or 5α-stanols are available commercially or can be generated using standard techniques (*e.g.*, Cholesterol- [1,2-^{3}H(N)], Cholesterol-[1,2,6,7-^{3}H(N)] or Cholesterol-[7-^{3}H(N)]; American Radiolabeled Chemicals, Inc; St. Louis, MO). In a preferred embodiment, ezetimibe is fluorescently labeled with a BODIPY group (Altmann, *et al.*, (2002) Biochim. Biophys. Acta 1580(1): 77-93) or labeled with a detectable group such as ^{35}S, ^{125}I or ^{3}H, preferably ^{35}S.

Direct Binding Assay. Typically, a given amount of NPC1L1 of the invention (*e.g.*, SEQ ID NO: 2, 4 or 12) or a complex including NPC1L1 is contacted with increasing amounts of labeled ligand or known antagonist or agonist (discussed above) and the amount of the bound, labeled ligand or known antagonist or agonist is measured after removing unbound, labeled ligand or known antagonist or agonist by washing. As the amount of the labeled ligand or known agonist or antagonist is increased, a point is eventually reached at which all receptor binding sites are occupied or saturated. Specific receptor binding of the labeled ligand or known agonist or antagonist is abolished by a large excess of unlabeled ligand or known agonist or antagonist.

Preferably, an assay system is used in which non-specific binding of the labeled ligand or known antagonist or agonist to the receptor is minimal. Non-specific binding is typically less than 50%, preferably less than 15%, more preferably less than 10%, and most preferably 5% or less, of the total binding of the labeled ligand or known antagonist or agonist.

In the basic binding assay, the method for identifying an NPC1L1 ligand, agonist or antagonist includes:

(a) contacting NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12), a fragment thereof or a complex including NPC1L1, in the presence of a known amount of labeled sterol (*e.g.*, cholesterol) or 5α-stanol or known antagonist or agonist (*e.g.*,

labeled ezetimibe) with a sample to be tested for the presence of an NPC1L1 ligand, agonist or antagonist; and

(b) measuring the amount of labeled sterol (*e.g.*, cholesterol) or 5α-stanol or known antagonist or agonist directly or indirectly bound to NPC1L1.

An NPC1L1 ligand in the sample is identified by measuring substantially reduced direct or indirect binding of the labeled sterol (*e.g.*, cholesterol) or 5α-stanol or known antagonist or agonist to NPC1L1, compared to what would be measured in the absence of such a ligand. For example, reduced direct or indirect binding between [^{3}H]-cholesterol and NPC1L1 in the presence of a sample might suggest that the sample contains a substance which is competing against [^{3}H]-cholesterol for NPC1L1 binding.

This assay can include a control experiment lacking any NPC1L1-dependent ligand (*e.g.*, sterol such as cholesterol or 5α-stanol) binding. In this assay, for example, a whole cell or cell membrane lacking any functional NPC1L1, for example, a cell or membrane isolated or derived from a transgenic mutant *npc1l1*$^-$ mouse of the invention, is assayed for ligand binding. When screening a sample for the presence of an NPC1L1 antagonist, it is useful to compare the level of binding observed in the presence of a sample being tested with that of a control experiment, as described herein, which completely lacks NPC1L1-dependent binding. Ideally, though by no means necessarily, the level of binding seen in the presence of a sample containing an antagonist will be similar to that of the control experiment.

Alternatively, a sample can be tested directly for binding to NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12). A basic assay of this type may include the following steps:

(a) contacting NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12), a fragment thereof or a complex including NPC1L1 with a labeled candidate compound (*e.g.*, [^{3}H]-ezetimibe); and

(b) detecting direct or indirect binding between the labeled candidate compound and NPC1L1.

Again, these experiment can be performed along with a control experiment wherein NPC1L1-dependent binding is completely lacking. For example, the assay can be performed using a whole cell or cell membrane lacking any

functional NPC1L1 (*e.g.*, cell or cell membrane derived from a transgenic, mutant *npc1l1*$^-$ mouse as described herein).

A candidate compound which is found to bind to NPC1L1 may function as ligand, agonist or antagonist of NPC1L1 (*e.g.*, by inhibition of sterol (*e.g.*, cholesterol) or 5α-stanol uptake).

In an embodiment of the invention, the bound candidate compound is quantified after filtration using glass fiber filters. In one aspect of this embodiment, the bound candidate compound is detected after single-tube vacuum filtration of GF/C glass fiber filters, obtained from Whatman. The filters may be pretreated by soaking with 0.5% polyethylenimine to reduce nonspecific binding. Filtration is accomplished by adding ice cold buffer to the assay tube, pouring the mixture through the filter, and then rinsing the tube and filter twice more with additional buffer. The buffer may be a Tris buffer or MES buffer (120 mM NaCl, 0.1% sodium cholate, and 20 mM MES at pH 6.70). The filters can be counted using scintillation fluid, e.g., Packard DM liquid or Packard Ultima Gold MV.

Alternatively, vacuum filtration of the sample on a Milliore 96-well plate (Whatman GF/C) can also be used to achieve adequate precision in a manner well-known to those skilled in the art.

SPA Assay. NPC1L1 ligands may also be measured using scintillation proximity assays (SPA). SPA assays are conventional and very well known in the art; see, for example, U.S. Patent No. 4,568,649. In SPA, the target of interest is immobilized to a small microsphere approximately 5 microns in diameter. The microsphere, typically, includes a solid scintillant core which has been coated with a polyhydroxy film, which in turn contains coupling molecules, which allow generic links for assay design. When a radioisotopically labeled molecule binds to the microsphere, the radioisotope is brought into close proximity to the scintillant and effective energy transfer from electrons emitted by the isotope will take place resulting in the emission of light. While the radioisotope remains in free solution, it is too distant from the scintillant and the electron will dissipate the energy into the aqueous medium and therefore remain undetected. Scintillation may be detected with a scintillation counter. In general, ^{3}H, ^{125}I and ^{35}S labels are well suited to SPA.

For the assay of receptor-mediated binding events, the lectin wheat germ agglutinin (WGA) may be used as the SPA bead coupling molecule (Amersham

Biosciences; Piscataway, NJ). The WGA coupled bead captures glycosylated, cellular membranes and glycoproteins and has been used for a wide variety of receptor sources and cultured cell membranes. The receptor is immobilized onto the WGA-SPA bead and a signal is generated on binding of an isotopically labeled ligand. Other coupling molecules which may be useful for receptor binding SPA assays include poly-L-lysine and WGA/polyethyleneimine (Amersham Biosciences; Piscataway, NJ). See, for example, Berry, J.A., *et al.*, (1991) Cardiovascular Pharmacol. 17 (Suppl.7): S143-S145; Hoffman, R., *et al.*, (1992) Anal. Biochem. 203: 70-75; Kienhus, *et al.*, (1992) J. Receptor Research 12: 389-399; Jing, S., *et al.*, (1992) Neuron 9: 1067-1079.

The scintillant contained in SPA beads may include, for example, yttrium silicate (YSi), yttrium oxide (YOx), diphenyloxazole or polyvinyltoluene (PVT) which acts as a solid solvent for diphenylanthracine (DPA).

SPA assays may be used to analyze whether a sample contains an NPC1L1 ligand. In these assays, a host cell which expresses NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12) on the cell surface or a membrane fraction thereof is incubated with and captured by SPA beads (*e.g.*, WGA coated YOx beads or WGA coated YSi beads). The beads bearing the NPC1L1 are incubated with labeled, known ligand or agonist or antagonist (*e.g.*, ^{3}H-cholesterol, ^{3}H-ezetimibe, ^{125}I-ezetimibe or a ^{35}S-ezetimibe analog). The assay mixture further includes either the sample to be tested or a blank (*e.g.*, water). After an optional incubation, scintillation is measured using a scintillation counter. An NPC1L1 ligand, agonist or antagonist may be identified in the sample by measuring substantially reduced fluorescence, compared to what would be measured in the absence of such ligand, agonist or antagonist (blank). Measuring substantially reduced fluorescence may suggest that the sample contains a substance which competes for direct or indirect NPC1L1 binding with the known ligand, agonist or antagonist.

Alternatively, a sample may be identified as an ligand of NPC1L1 by directly detecting binding in a SPA assay. In this assay, a labeled version of a candidate compound to be tested may be put in contact with the host cell expressing NPC1L1 or a membrane fraction thereof which is bound to the SPA bead. Fluorescence may then be assayed to detect the presence of a complex between the labeled candidate compound and the host cell or membrane fraction expressing

NPC1L1 or a complex including NPC1L1. A candidate compound which binds directly or indirectly to NPC1L1 may possess NPC1L1 agonistic or antagonistic activity.

SPA Assays can also be performed along with a control experiment lacking any NPC1L1-dependent binding. The control experiment can be performed, for example, with a cell or cell membrane lacking any functional NPC1L1 (*e.g.*, cell or cell membrane derived from a transgenic, mutant npc111- mouse as described herein). When the control experiment is performed, the level of binding observed in the presence of sample being tested for the presence of an antagonist can be compared with that observed in the control experiment.

Sterol/5α-stanol Uptake Assay. Assays may also be performed to determine if a sample can agonize or antagonize NPC1L1 mediated sterol (*e.g.*, cholesterol) or 5α-stanol uptake. In these assays, a host cell expressing NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12) on the cell surface (discussed above) can be contacted with detectably labeled sterol (*e.g.*, ^{3}H-cholesterol or ^{125}I-cholesterol)) or 5α-stanol along with either a sample or a blank. After an optional incubation, the cells can be washed to remove unabsorbed sterol or 5α-stanol. Sterol or 5α-stanol uptake can be determined by detecting the presence of labeled sterol or 5α-stanol in the host cells. For example, assayed cells or lysates or fractions thereof (*e.g.*, fractions resolved by thin-layer chromatography) can be contacted with a liquid scintillant and scintillation can be measured using a scintillation counter.

In these assays, an NPC1L1 antagonist in the sample may be identified by measuring substantially reduced uptake of labeled sterol (*e.g.*, ^{3}H-cholesterol) or 5α-stanol, compared to what would be measured in the absence of such an antagonist and an agonist may be identified by measuring substantially increased uptake of labeled sterol (*e.g.*, ^{3}H-cholesterol) or 5α-stanol, compared to what would be measured in the absence of such an agonist.

Uptake assays can also be performed along with a control experiment lacking any NPC1L1-dependent uptake. The control experiment can be performed, for example, with a cell lacking any functional NPC1L1 (*e.g.*, cell derived from a transgenic, mutant *npc111*$^{-}$ mouse as described herein). When the control experiment is performed, the level of uptake observed in the presence of sample being tested for

the presence of an antagonist can be compared with that observed in the control experiment.

Source of NPC1L1. In principle, a binding assay of the invention could be carried out using a soluble NPC1L1 polypeptide of the invention, *e.g.*, following production and refolding by standard methods from an *E. coli* or other prokaryotic or eukaryotic expression system, and the resulting receptor-labeled ligand complex could be precipitated, *e.g.*, using an antibody against the receptor. The precipitate could then be washed and the amount of the bound, labeled ligand or antagonist or agonist could be measured.

Alternatively, NPC1L1 is membrane-bound. A nucleic acid encoding an NPC1L1 polypeptide of the invention (*e.g.*, SEQ ID NO: 2, 4 or 12) can be transfected into an appropriate host cell, whereby the NPC1L1 will become incorporated into the membrane of the cell. A membrane fraction can then be isolated from the cell and used as a source of NPC1L1 for assay. Alternatively, the whole cell expressing NPC1L1 in the cell surface can be used in an assay. Preferably, specific binding of the labeled ligand or known antagonist or agonist to an untransfected/untransformed host cell or to a membrane fraction from an untransfected/untransformed host cell will be negligible.

Various membranes may be used directly as a source of NPC1L1 for the above-described screening systems, e.g. direct binding, scintillation proximity assay, sterol/5α stanol uptake assay. As described in Examples 5, 6, 7, 8, 9, 17, 27, and 29, NPC1L1 is highly expressed in certain tissues, especially in brush border cells of intestinal tissues. Therefore, brush border membrane (BBM) vesicle preparations may be utilized as a source of NPC1L1. The membranes may be derived from mammalian intestinal tissue from rhesus, rat, mouse or human tissue.

Membranes may be derived from brush border cells of intestinal tissues. Such membranes are conventionally prepared by collecting intestinal tissue from freshly sacrificed animals. The mucosa of the tissue is scraped, collected into buffered solutions, and homogenized. Cellular debris is removed and the membrane fractions are collected by centrifugation. Conventional techniques known to one of skill in the art maybe used for the preparation of brush border membrane vesicles. *See* Hauser, H., Howell, K., Dawson, R.M.C., Bowyer, D. E. Biochim. Biophys. Acta 602, 567-577 (1980); Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal,

K., Muller, G., Tripier, D., Wess, G. J. Biol. Chem. 268, 18035-18046 (1993); Rigtrup, K.M., Ong, D.E. Biochemistry 31, 2920-2926 (1992).

The membrane preparation may be in vesicular or non-vesicular form. Alternatively, liposomes and liposomal preparations comprising NPC1L1 may also be a viable source of NPC1L1 for the screening assays of the present claimed method.

In vitro cultured cells expressing NPC1L1 may also be used. The host cells may be prepared by transforming or transfecting a nucleic acid encoding an NPC1L1 of the invention into an appropriate host cell, whereby the receptor becomes incorporated into the membrane of the cell. A membrane fraction can then be isolated from the cell and used as a source of the receptor for assay. Alternatively, the whole cell expressing the receptor on the cell surface can be used in an assay. Preferably, specific binding of the labeled ligand or known antagonist or agonist to an untransfected/untransformed host cell or membrane fraction from an untransfected/untransformed host cell will be negligible.

Preferred host cells include Chinese Hamster Ovary (CHO) cells, murine macrophage J774 cells, HEK-293 cells or any other macrophage cell line and human intestinal epithelial Caco2 cells.

The present invention provides for a method of identifying a ligand of NPC1L1 using these membrane preparations, for example by contacting membranes comprising NPC1L1, such as brush border membrane vesicle preparations, with detectably labeled substituted azetidinone compounds which are known NPC1L1 ligands, agonists or antagonists, and a candidate compound and determining whether the candidate compound can bind to NPC1L1. The binding of the candidate compound to NPC1L1 may modulate binding of the detectably labeled NPC1L1 ligands, agonists or antagonists to NPC1L1. In addition, a NPC1L1 ligand may be identified by measuring the binding of NPC1L1 with detectably labeled NPC1L1 ligands, agonists or antagonists in the presence and absence of the candidate compound wherein decreased binding of the detectably labeled NPC1L1 ligands, agonists or antagonists to NPC1L1 is an indication that the candidate compound is ligand of NPC1L1.

NPC1L1 may also be obtained by solubilization of membrane fractions comprising NPC1L1. The membranes may be obtained as discussed above, *e.g.,* from mammalian tissue or *in vitro* cultured cells.

Binding Affinities of NPC1L1 Ligands. The affinity and specificity of the known ligand (e.g., detectably labeled substituted 2-azetidinone-glucuronide) for NPC1L1 are important to the identification of ligands that bind NPC1L1 in a screening assay. It is understood that the known ligand will be labeled for use in the screening assay. In an embodiment of the invention, the binding affinity of the known ligand for human NPC1L1 has a K_D value equivalent or lower than the K_D value of ezetimibe glucuronide **1** for human NPC1L1. In an aspect of this embodiment, the binding affinity of the known ligand for human NPC1L1 has a K_D value of about 200nM or lower; particularly it has a K_D value of about 100nM or lower; more particularly it has a K_D value of about 50nM or lower; even more particularly it has a K_D value of about 20nM or lower; and most particularly it has a K_D value of about 10nM or lower. For usefulness in the assay, there is essentially no lower limit on the K_D value of the known ligand and it may, for example, go down into the pM range. As the K_D value decreases, the binding affinity of the ligand for human NPC1L1 increases, which is desirable for the screening assay.

In another embodiment of the invention, the binding affinity of the known ligand for rat NPC1L1 has a K_D value equivalent or lower than the K_D value of ezetimibe glucuronide **1** for rat NPC1L1. In an aspect of this embodiment, the binding affinity of the known ligand for rat NPC1L1 has a K_D value of about 200nM or lower; particularly it has a K_D value of about 100nM or lower; more particularly it has a K_D value of about 50nM or lower; even more particularly it has a K_D value of about 20nM or lower; and most particularly it has a K_D value of about 10nM or lower.

In another embodiment of this invention, the known ligand for human NPC1L1 is selected from (a) a sulfur-containing substituted 2-azetidinone-glucuronide that is labeled with ^{35}S, and particularly a compound of Formula II wherein R^9 contains an $-SO_2-$ group and (b) a substituted 2-azetidinone-glucuronide labeled with ^{125}I.

In one aspect of this embodiment, the known ligand for human NPC1L1 is selected from (a) a sulfur-containing substituted 2-azetidinone-glucuronide that is labeled with ^{35}S, and particularly a compound of Formula II

wherein R^9 contains an -SO_2- group, and (b) a substituted 2-azetidinone-glucuronide labeled with ^{125}I, and has a K_D value equivalent or lower than the K_D value of ezetimibe glucuronide 1.

In another aspect of this embodiment, the known ligand for human NPC1L1 is selected from (a) a sulfur-containing substituted 2-azetidinone-glucuronide that is labeled with ^{35}S, and particularly a compound of Formula II wherein R^9 contains an -SO_2- group, and (b) a substituted 2-azetidinone-glucuronide labeled with ^{125}I, and has a K_D value of about 200nM or lower; particularly it has a K_D value of about 100nM or lower; more particularly it has a K_D value of about 50nM or lower; even more particularly it has a K_D value of about 20nM or lower; and most particularly it has a K_D value of about 10nM or lower.

When using ^3H-labeled ezetimibe glucuronide in screening to identify NPC1L1 ligands from among the candidate compounds using mouse-derived membranes, candidate compounds identified as NPC1L1 ligands are preferably those candidates that exhibit a binding affinity having a K_D value of about 12,000nM or lower, preferably about 1000nM or lower, more preferably about 100nM or lower, and most preferably about 10nM or lower. When using ^3H-labeled ezetimibe glucuronide in screening to identify NPC1L1 ligands using rat-derived membranes or human-derived membranes, candidate compounds identified as NPC1L1 ligands are preferably those candidates that exhibit a binding affinity having a K_D value of about 1000nM or lower, preferably about 100nM or lower, and more preferably about 10nM or lower. When using ^3H-labeled ezetimibe glucuronide in screening to identify NPC1L1 ligands using rhesus monkey-derived membranes, candidate compounds identified as NPC1L1 ligands are preferably those candidates that exhibit a binding affinity having a K_D value of about 50nM or lower, and preferably about 10nM or lower.

When using ^{35}S-labeled compound 2 in screening to identify NPC1L1 ligands from among the candidate compounds using rat or human-derived membranes, candidate compounds identified as NPC1L1 ligands are preferably those candidates that exhibit a binding affinity having a K_D value in the range from about 10μM to about 1nM. When using ^{125}I-labeled substituted 2-azetidinone glucuronide compounds in the assay with rat or human membranes, candidate compounds identified as NPC1L1 ligands are preferably those candidates that exhibit a binding

affinity having a K_D value in the range from about 10nM to about 10pM, and preferably from about 100pM to about 10pM.

Mouse Assay. The present invention comprises a mutant, transgenic mouse which lacks any functional NPC1L1. This mouse may serve as a convenient control experiment in screening assays for identifying inhibitors of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption, preferably inhibitors of NPC1L1. Preferably, a mouse assay of the present invention would comprise the following steps:

(a) feeding a sterol (*e.g.*, cholesterol) or 5α-stanol-containing substance (*e.g.*, comprising radiolabeled cholesterol, such as ^{14}C-cholesterol or ^{3}H-cholesterol) to a first and second mouse comprising a functional *NPC1L1* gene and to a third, mutant mouse lacking a functional NPC1L1;

The sterol (*e.g.*, cholesterol) or 5α-stanol containing substance preferably contains labeled cholesterol, such as a radiolabeled cholesterol, for example, ^{3}H or ^{14}C labeled cholesterol. The sterol (*e.g.*, cholesterol) or 5α-stanol containing substance may also include cold, unlabeled sterol (*e.g.*, cholesterol) or 5α-stanol such as in corn oil.

In these assays, the third *npc1l1*$^{-}$ mutant mouse serves as a (+)-control experiment which exhibits low levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption and the second mouse serves as a (-)-control experiment which exhibits normal, uninhibited levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption. The second mouse is not administered the sample to be tested for an NPC1L1 antagonist. The first mouse is the experiment.

(b) administering the sample to the first mouse comprising a functional *NPC1L1* but not to the second mouse;

(c) measuring the amount of sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the intestine of said first, second and third mouse;

Intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption may be measured by any method known in the art. For example, the level intestinal absorption can be assayed by measuring the level of serum sterol (*e.g.*, cholesterol) or 5α-stanol.

(d) comparing the levels of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in each mouse;

wherein the sample is determined to contain the intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption antagonist when the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the first mouse and in the third mouse are less than the amount of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the second mouse.

Preferably, if the sample contains an intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption inhibitor (*e.g.*, an NPC1L1 inhibitor), the level of sterol (*e.g.*, cholesterol) or 5α-stanol absorption in the first mouse will be similar to that of the third, *npc1l1* mutant mouse.

An alternative, (+)-control experiment which may be used in these screening assays is a mouse comprising functional NPC1L1 which is administered a known antagonist of NPC1L1, such as ezetimibe.

Pharmaceutical Compositions

NPC1L1 ligands discovered, for example, by the screening methods described above may be used therapeutically (*e.g.*, in a pharmaceutical composition) to stimulate or block the activity of NPC1L1 and, thereby, to treat any medical condition caused or mediated by NPC1L1. In addition, the antibody molecules of the invention may also be used therapeutically (*e.g.*, in a pharmaceutical composition) to bind NPC1L1 and, thereby, block the ability of NPC1L1 to bind a sterol (*e.g.*, cholesterol) or 5α-stanol. Blocking the binding of a sterol (*e.g.*, cholesterol) or 5α-stanol would prevent absorption of the molecule (*e.g.*, by intestinal cells such as enterocytes). Blocking absorption of sterol (*e.g.*, cholesterol) or 5α-stanol would be a useful way to lower serum sterol (*e.g.*, cholesterol) or 5α-stanol levels in a subject and, thereby, reduce the incidence of, for example, hyperlipidemia, atherosclerosis, coronary heart disease, stroke or arteriosclerosis.

The term "subject" or "patient" includes any organism, preferably animals, more preferably mammals (*e.g.*, mice, rats, rabbits, dogs, horses, primates, cats) and most preferably humans.

The term "pharmaceutical composition" refers to a composition including an active ingredient and a pharmaceutically acceptable carrier and/or adjuvant.

Although the compositions of this invention could be administered in simple solution, they are more typically used in combination with other materials such

as carriers, preferably pharmaceutically acceptable carriers. Useful, pharmaceutically acceptable carriers can be any compatible, non-toxic substances suitable for delivering the compositions of the invention to a subject. Sterile water, alcohol, fats, waxes, and inert solids may be included in a pharmaceutically acceptable carrier. Pharmaceutically acceptable adjuvants (buffering agents, dispersing agents) may also be incorporated into the pharmaceutical composition.

Preferably, the pharmaceutical compositions of the invention are in the form of a pill or capsule. Methods for formulating pills and capsules are very well known in the art. For example, for oral administration in the form of tablets or capsules, the active drug component may be combined with any oral, non-toxic pharmaceutically acceptable inert carrier, such as lactose, starch, sucrose, cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, talc, mannitol, ethyl alcohol (liquid forms) and the like. Moreover, when desired or needed, suitable binders, lubricants, disintegrating agents and coloring agents may also be incorporated in the mixture. Suitable binders include starch, gelatin, natural sugars, corn sweeteners, natural and synthetic gums such as acacia, sodium alginate, carboxymethylcellulose, polyethylene glycol and waxes. Among the lubricants there may be mentioned for use in these dosage forms, boric acid, sodium benzoate, sodium acetate, sodium chloride, and the like. Disintegrants include starch, methylcellulose, guar gum and the like. Sweetening and flavoring agents and preservatives may also be included where appropriate.

The pharmaceutical compositions of the invention may be administered in conjunction with a second pharmaceutical composition or substance. In preferred embodiments, the second composition includes a cholesterol-lowering drug. When a combination therapy is used, both compositions may be formulated into a single composition for simultaneous delivery or formulated separately into two or more compositions (*e.g.*, a kit).

The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. See, *e.g.*, Gilman *et al.* (eds.) (1990), *The Pharmacological Bases of Therapeutics,* 8th Ed., Pergamon Press; and *Remington's Pharmaceutical Sciences, supra,* Easton, Penn.; Avis *et al.* (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications* Dekker, New York; Lieberman *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms:*

Tablets Dekker, New York; and Lieberman *et al.* (eds.) (1990), *Pharmaceutical Dosage Forms: Disperse Systems* Dekker, New York.

The dosage regimen involved in a therapeutic application may be determined by a physician, considering various factors which may modify the action of the therapeutic substance, *e.g.*, the condition, body weight, sex and diet of the patient, the severity of any infection, time of administration, and other clinical factors. Often, treatment dosages are titrated upward from a low level to optimize safety and efficacy. Dosages may be adjusted to account for the smaller molecular sizes and possibly decreased half-lives (clearance times) following administration.

An "effective amount" of a ligand of the invention may be an amount that will detectably reduce the level of intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption or detectably reduce the level of serum sterol (*e.g.*, cholesterol) or 5α-stanol in a subject administered the composition.

Typical protocols for the therapeutic administration of such substances are well known in the art. Pharmaceutical composition of the invention may be administered, for example, by any parenteral or non-parenteral route.

Pills and capsules of the invention can be administered orally. Injectable compositions can be administered with medical devices known in the art; for example, by injection with a hypodermic needle.

Injectable pharmaceutical compositions of the invention may also be administered with a needleless hypodermic injection device; such as the devices disclosed in U.S. Patent Nos. 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824 or 4,596,556.

Anti-Sense

The present invention also encompasses anti-sense oligonucleotides capable of specifically hybridizing to mRNA encoding NPC1L1 (*e.g.*, any of SEQ ID NOs: 1, 3, 5-11 or 13) having an amino acid sequence defined by, for example, SEQ ID NO: 2 or 4 or 12 or a subsequence thereof so as to prevent translation of the mRNA. Additionally, this invention contemplates anti-sense oligonucleotides capable of specifically hybridizing to the genomic DNA molecule encoding NPC1L1, for example, having an amino acid sequence defined by SEQ ID NO: 2 or 4 or 12 or a subsequence thereof.

This invention further provides pharmaceutical compositions comprising (a) an amount of an oligonucleotide effective to reduce NPC1L1-mediated sterol (*e.g.*, cholesterol) or 5α-stanol absorption by passing through a cell membrane and binding specifically with mRNA encoding NPC1L1 in the cell so as to prevent its translation and (b) a pharmaceutically acceptable carrier capable of passing through a cell membrane. In an embodiment, the oligonucleotide is coupled to a substance that inactivates mRNA. In another embodiment, the substance that inactivates mRNA is a ribozyme.

Reducing the level of NPC1L1 expression by introducing anti-sense NPC1L1 RNA into the cells of a patient is a useful method reducing intestinal sterol (*e.g.*, cholesterol) or 5□-stanol absorption and serum cholesterol in the patient.

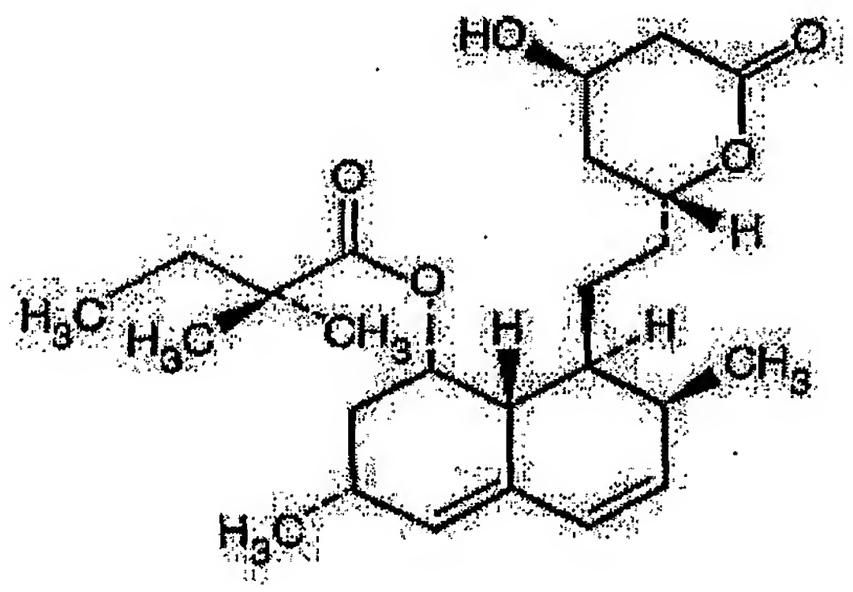
Kits

Kits of the present invention include ezetimibe, preferably combined with a pharmaceutically acceptable carrier, in a pharmaceutical formulation, more preferably in a pharmaceutical dosage form such as a pill, a powder, an injectable liquid, a tablet, dispersible granules, a capsule, a cachet or a suppository. See for example, Gilman *et al.* (eds.) (1990), The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, supra, Easton, Penn.; Avis *et al.* (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications Dekker, New York; Lieberman *et al.* (eds.) (1990) Pharmaceutical Dosage Forms: Tablets Dekker, New York; and Lieberman *et al.* (eds.) (1990), Pharmaceutical Dosage Forms: Disperse Systems Dekker, New York. Preferably, the dosage form is a Zetia® tablet (Merck/Schering-Plough Corp.). Ezetimibe may be supplied in any convenient form. For example, tablets including ezetimibe may be supplied in bottles of 30, 90 or 500.

The kits of the present invention also include information, for example in the form of a package insert, indicating that the target of ezetimibe is NPC1L1 (NPC3). The term "target of ezetimibe" indicates that ezetimibe reduces intestinal sterol (*e.g.*, cholesterol) or 5α-stanol absorption, either directly or indirectly, by antagonizing NPC1L1. The form of the insert may take any form, such as paper or on electronic media such as a magnetically recorded medium (*e.g.*, floppy disk) or a CD-ROM.

The package insert may also include other information concerning the pharmaceutical compositions and dosage forms in the kit. Generally, such information aids patients and physicians in using the enclosed pharmaceutical compositions and dosage forms effectively and safely. For example, the following information regarding ezetimibe (*e.g.*, Zetia®) and/or simvastatin (*e.g.*, Zocor®) may be supplied in the insert: pharmacokinetics, pharmacodynamics, clinical studies, efficacy parameters, indications and usage, contraindications, warnings, precautions, adverse reactions, overdosage, proper dosage and administration, how supplied, proper storage conditions, references and patent information.

The kits of the invention may also include simvastatin (



) preferably combined with a pharmaceutically acceptable carrier, in a pharmaceutical formulation, more preferably in a pharmaceutical dosage form such as a pill, a powder, an injectable liquid, a tablet, dispersible granules, a capsule, a cachet or a suppository. Preferably, the dosage form of simvastatin is a Zocor® tablet (Merck & Co.; Whitehouse Station, NJ).

Tablets or pills comprising simvastatin may be supplied in any convenient form. For example, pills or tablets comprising 5mg simvastatin can be supplied as follows: bottles of 30, 60, 90, 100 or 1000. Pills or tablets comprising 10 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000. Pills or tablets comprising 20 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000. Pills or tablets comprising 40 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100 or 1000. Pills or tablets comprising 80 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000.

Ezetimibe and simvastatin may be supplied, in the kit, as separate compositions or combined into a single composition. For example, ezetimibe and simvastatin may be supplied within a single, common pharmaceutical dosage form (*e.g.*, pill or tablet) as in separate pharmaceutical dosage forms (*e.g.*, two separate pills or tablets).

npc1l1⁻ Cells

The present invention provides any isolated mammalian cell, (*e.g.*, an isolated mouse cell, an isolated rat cell or an isolated human cell) which lacks an NPC1L1 gene which encodes or can produce a functional NPC1L1 protein. Included within this embodiment are mutant *npc1l1* genes comprising a point mutation, truncation or deletion of the genetic coding region or of any regulatory element (*e.g.*, a promoter).

For example, the cell can be isolated from a mutant mouse comprising a homozygous mutation of endogenous, chromosomal NPC1L1 wherein the mouse does not produce any functional NPC1L1 protein (*e.g.*, the mouse described below in Example 22). Moreover, the present invention comprises any cell, tissue, organ, fluid, nucleic acid, peptide or other biological substance derived or isolated from such a mutant mouse, particularly a mutant, transgenic mouse which does not produce any functional NPC1L1, wherein the region of endogenous, chromosomal NPC1L1 deleted, in the mouse, corresponds to nucleotides 790-998 of the nucleotide sequence set forth in SEQ ID NO: 45.

The isolated cell can be isolated or derived, for example, from the duodenum, gall bladder, liver, small intestine or stomach of the mutant mouse. Further, the cell can be an enterocyte.

The *npc1l1*⁻ mutant cells are useful, for example, for use in control experiments in screening assays (see *e.g.*, supra) since they lack any NPC1L1-dependent uptake or binding of sterol, 5α-stanol or ezetimibe. The level of inhibition caused by a particular sample, in a screening assay, can be compared to that of an assay performed with the mutant cell. Ideally, though by no means necessarily, in a screening assay, for example, as described herein, the same amount of binding will be observed by a non-mutant cell or cell membrane, in the presence of an antagonist, as is observed in connection with a mutant *npc1l1* cell or cell membrane alone.

EXAMPLES

The following examples are provided to more clearly describe the present invention and should not be construed to limit the scope of the invention in any way.

Example 1: Cloning and Expression of Rat, Mouse and Human NPC1L1.

Rat *NPC1L1*, mouse *NPC1L1* or human *NPC1L1* can all conveniently be amplified using polymerase chain reaction (PCR). In this approach, DNA from a rat, mouse or human cDNA library can be amplified using appropriate primers and standard PCR conditions. Design of primers and optimal amplification conditions constitute standard techniques which are commonly known in the art.

An amplified *NPC1L1* gene may conveniently be expressed, again, using methods which are commonly known in the art. For example, NPC1L1 may be inserted into a pET-based plasmid vector (Stratagene; La Joola, CA), downstream of the T7 RNA polymerase promoter. The plasmid may then be transformed into a T7 expression system (*e.g.*, BL21DE3 E.coli cells), grown in a liquid culture and induced (*e.g.*, by adding IPTG to the bacterial culture).

Example 2: Direct Binding Assay.

Membrane preparation: Caco2 cells transfected with an expression vector containing a polynucleotide encoding NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12) are harvested by incubating in 5 mM EDTA/phosphate-buffered saline followed by repeated pipeting. The cells are centrifuged 5 min at 1000 x g. The EDTA/PBS is decanted and an equal volume of ice-cold 50mM Tris-HCl, pH 7.5 is added and cells are broken up with a Polytron (PT10 tip, setting 5, 30 sec). Nuclei and unbroken cells are sedimented at 1000 x g for 10 min and then the supernatant is centrifuged at 50,000 x g for 10 min. The supernatant is decanted, the pellet is resuspended by Polytron, a sample is taken for protein assay (bicinchoninic acid, Pierce), and the tissue is again centrifuged at 50,000 x g. Pellets are stored frozen at –20°C.

Binding assay: For saturation binding, four concentrations of [^{3}H]-ezetimibe (15 Ci/mmol) are incubated without and with 10^{-5} M ezetimibe in triplicate with 50 µg of membrane protein in a total volume of 200 µl of 50 mM Tris-HCl, pH 7.5, for 30 min at 30°C. Samples are filtered on GF/B filters and washed three times with 2 ml of cold Tris buffer. Filters are dried in a microwave oven, impregnated

with Meltilex wax scintillant, and counted at 45% efficiency. For competition binding assays, five concentrations of a sample are incubated in triplicate with 18 nM [^{3}H]-ezetimibe and 70 μg of membrane protein under the conditions described above. Curves are fit to the data with Prism (GraphPad Software) nonlinear least-squares curve-fitting program and K_i values are derived from IC_{50} values according to Cheng and Prusoff (Cheng, Y. C., *et al.,* (1973) Biochem. Pharmacol. 22: 3099-3108).

Example 3A: SPA Assay.

For each well of a 96 well plate, a reaction mixture of 10 μg human, mouse or rat NPC1L1-CHO overexpressing membranes (Biosignal) and 200 μg/well YSi-WGA-SPA beads (Amersham) in 100 μl is prepared in NPC1L1 assay buffer (25 mM HEPES, pH 7.8, 2 mM $CaCl_2$, 1mM $MgCl_2$, 125 mM NaCl, 0.1% BSA). A 0.4 nM stock of ligand- [^{125}I]-ezetimibe- is prepared in the NPC1L1 assay buffer. The above solutions are added to a 96-well assay plate as follows: 50 μl NPC1L1 assay buffer, 100 μl of reaction mixture, 50 μl of ligand stock (final ligand concentration is 0.1 nM). The assay plates are shaken for 5 minutes on a plate shaker, then incubated for 8 hours before cpm/well are determined in Microbeta Trilux counter (PerkinElmer).

These assays will indicate that [^{125}I]-ezetimibe binds to the cell membranes expressing human, mouse or rat NPC1L1. Similar results will be obtained if the same experiment is performed with radiolabeled cholesterol (*e.g.*, ^{125}I-cholesterol).

Example 3A: Alternate SPA Assay.

The final concentrations should be: 1 nM ^{35}S-**2** (Km ~ 2-5 nM, ~50,000 dpm/assay);

1 μg membranes (~1-2 nM receptor); 0.007%-0.03% taurocholate (0.140 μl 1% stock); 0.010%-0.05% digitonin (0.200 μl 1% stock); 5% DMSO (1.00 μl inhibitors).

In each well of a 96 well plate is put 1 μl DMSO inhibitor solution, and then the radioligand and detergents are added as a 2X solution in 10 μl buffer A. Shake for a minute to be sure the inhibitor and ligand are mixed, before initiating with 9 μl diluted receptor solution in buffer A. After shaking again, the plate is incubated at 37°C for 2 hours. Then WGA beads (0.3 mg) are added as a 3 μl suspension in

buffer A, then shake for 30 minutes. Similar results are obtained if membranes are pre-incubated with beads for 30 minutes before adding ligands. Finally, dilute to 300 µl with buffer A, cover the plate, spin at 3,000 rpmx5 min, and read at 2 minutes per well in the "Microbeta" counter.

Stocks

Ligand: ^{35}S-**2** is 525.42 nM, 0616 µCi/µl, in acetonitrile; spec. act. = 3.8916 X 10-^{4} fmol/dpm; 1168 Ci/mmol)

Membranes: 3rd batch recombinant human expressed in HEK-293 cells; 20.2 ug/µl stock; ~20–40 pmol NPC1L1/mg protein

Buffer A: 26 mM $NaHCO_3$; 0.96 mN NaH_2PO_4; 5 mM HEPES; optional addition of 5.5 mM glucose; 117 mM NaCl; 5.4 mM KC1

Example 4: Cholesterol Uptake Assay.

CHO cells expressing either SR-B1 or three different clones of rat NPC1L1 or one clone of mouse NPC1 L1 were starved overnight in cholesterol free media then dosed with [3H]-cholesterol in a mixed synthetic micelle emulsion for 4 min, 8 min, 12 min or 24 min in the absence or presence of 10 □M ezetimibe. The cells were harvested and the lipids were organically extracted. The extracted lipids were spotted on thin-layer chromatography (TLC) plates and resolved within an organic vapor phase. The free cholesterol bands for each assay were isolated and counted in a scintillation counter.

The SR-B1 expressing cells exhibited an increase in [^{3}H]-cholesterol uptake as early as 4 min which was also inhibited by ezetimibe. The three rat clones and the one mouse clone appeared to give background levels of [^{3}H]-cholesterol uptake which was similar to that of the untransformed CHO cell.

These experiments will yield data demonstrating that CHO cells can perform mouse, rat and human NPC1L1-dependent uptake of [^{3}H]-cholesterol when more optimal experimental conditions are developed.

Example 5: Expression of Rat NPC1L1 in Wistar Rat Tissue.

In these experiments, the expression of rat *NPC1L1* mRNA, in several rat tissues, was evaluated. The tissues evaluated were esophagus, stomach, duodenum, jejunum, ileum, proximal colon, distal colon, liver, pancreas, heart, aorta, spleen, lung, kidney, brain, muscle, testes, ovary, uterus, adrenal gland and

thyroid gland. Total RNA samples were isolated from at least 3 male and 3 female animals and pooled. The samples were then subjected to real time quantitative PCR using Taqman analysis using standard dual-labeled fluorogenic oligonucleotide probes. Typical probe design incorporated a 5' reporter dye (*e.g.*, 6FAM (6-carboxyfluorescein) or VIC) and a 3' quenching dye (*e.g.*, TAMRA (6-carboxytetramethyl-rhodamine)).

rat *NPC1L1*:

Forward: TCTTCACCCTTGCTCTTTGC (SEQ ID NO: 14)

Reverse: AATGATGGAGAGTAGGTTGAGGAT (SEQ ID NO: 15)

Probe: [6FAM]TGCCCACCTTTGTTGTCTGCTACC[TAMRA] (SEQ ID NO: 16) rat β-actin:

Forward: ATCGCTGACAGGATGCAGAAG (SEQ ID NO: 17)

Reverse: TCAGGAGGAGCAATGATCTTGA (SEQ ID NO: 18)

Probe: [VIC]AGATTACTGCCCTGGCTCCTAGCACCAT[TAMRA] (SEQ ID NO: 19)

PCR reactions were run in 96-well format with 25 µl reaction mixture in each well containing: Platinum SuperMix (12.5 µl), ROX Reference Dye (0.5 µl), 50 mM magnesium chloride (2 µl), cDNA from RT reaction (0.2 µl). Multiplex reactions contained gene specific primers at 200 nM each and FAM labeled probe at 100 nM and gene specific primers at 100 nM each and VIC labeled probe at 50 nM. Reactions were run with a standard 2-step cycling program, 95°C for 15 sec and 60°C for 1 min, for 40 cycles.

The highest levels of expression were observed in the duodenum, jejunum and ileum tissue. These data indicate that NPC1L1 plays a role in cholesterol absorption in the intestine.

Example 6: Expression of Mouse NPC1L1 in Mouse Tissue.

In these experiments, the expression of mouse *NPC1L1* mRNA, in several tissues, was evaluated. The tissues evaluated were adrenal gland, BM, brain, heart, islets of langerhans, LI, small intestine, kidney, liver, lung, MLN, PLN, muscle, ovary, pituitary gland, placenta, Peyers Patch, skin, spleen, stomach, testes, thymus, thyroid gland, uterus and trachea. Total RNA samples were isolate from at least 3 male and 3 female animals and pooled. The samples were then subjected to real time quantitative PCR using Taqman analysis using the following primers and probes:

mouse *NPC1L1*:

Forward: ATCCTCATCCTGGGCTTTGC (SEQ ID NO: 20)

Reverse: GCAAGGTGATCAGGAGGTTGA (SEQ ID NO: 21)

Probe: [6FAM]CCCAGCTTATCCAGATTTTCTTCTTCCGC[TAMRA] (SEQ ID NO: 22)

The highest levels of expression were observed in the Peyer's Patch, small intestine, gall bladder and stomach tissue. These data are consistent with a cholesterol absorption role for NPC1L1 which takes place in the digestive system.

Example 7: Expression of Human NPC1L1 in Human Tissue.

In these experiments, the expression level of human NPC1L1 mRNA was evaluated in 2045 samples representing 46 normal tissues. Microarray-based gene expression analysis was performed on the Affymetrix HG-U95 GeneChip using a cRNA probe corresponding to base pairs 4192-5117 (SEQ ID NO: 43) in strict accordance to Affymetrix's established protocols. Gene Chips were scanned under low photo multiplier tube (PMT), and data were normalized using either Affymetrix MAS 4.0 or MAS 5.0 algorithms. In addition "spike ins" for most samples were used to construct a standard curve and obtain RNA concentration values according Gene Logic algorithms and procedures. A summary of these results are indicated, below, in Table 2.

Table 2. Expression level of *NPC1L1* mRNA in various human tissues.

Tissue	Present	Absent	Lower 25%	Median	Upper 75%
Adipose	2 of 32	30 of 32	-2.45	1.16	12.23
Adrenal Gland	0 of 12	12 of 12	-23.54	-4.47	10.51
Appendix	0 of 3	3 of 3	-8.02	-6.69	38.19
Artery	0 of 3	3 of 3	-6.59	-4.67	9.68
Bladder	1 of 5	4 of 5	-22	-7.95	-1.99
Bone	0 of 3	3 of 3	-1.64	3.3	19.53
Breast	4 of 80	76 of 80	-4.07	3.13	14.67
Cerebellum	0 of 5	5 of 5	-3.04	3.24	15.38
Cervix	3 of 101	98 of 101	-7.56	-0.07	20.89
Colon	9 of 151	142 of 151	-10.19	0.31	18.36
Cortex Frontal Lobe	0 of 7	7 of 7	1.4	8.46	11.75
Cortex Temporal Lobe	0 of 3	3 of 3	7.1	8.5	15.87
Duodenum	59 of 61	2 of 61	519.23	827.43	1101.67
Endometrium	0 of 21	21 of 21	-14.43	-6.39	2.79
Esophagus	1 of 27	26 of 27	-10.93	-4.97	12.48
Fallopian Tube	3 of 51	48 of 51	5.02	13.24	26.77
GallBladder	8 of 8	0 of 8	205.76	273.39	422.8
Heart	0 of 3	3 of 3	3.33	11.19	11.66
Hippocampus	0 of 5	5 of 5	8.25	9.11	19.83
Kidney	4 of 86	82 of 86	-8.36	3.41	16.46
Larynx	0 of 4	4 of 4	-13.76	-0.81	8.54
Left Atrium	2 of 141	139 of 141	-18.9	-4.58	6.84
Left Ventricle	0 of 15	15 of 15	-21.19	-9.59	17.7
Liver	32 of 34	2 of 34	325.74	427.77	540.1
Lung	2 of 93	91 of 93	-3.47	11.03	22.34
Lymph Node	0 of 11	11 of 11	-1.78	-0.19	1.34
Muscles	0 of 39	39 of 39	-21.57	8.25	26.73
Myometrium	8 of 106	98 of 106	-3.98	4.87	17.55
Omentum	0 of 15	15 of 15	-14.25	-1.6	19.58
Ovary	1 of 74	73 of 74	0.5	17.51	38.28
Pancreas	0 of 34	34 of 34	-87.08	-53.2	-24.14
Placenta	0 of 5	5 of 5	-20.4	-3.44	18.91
Prostate	0 of 32	32 of 32	1.08	15.56	27.24
Rectum	1 of 43	42 of 43	-9.26	-1.49	9.8
Right Atrium	4 of 169	165 of 169	-19.32	-6.58	7.72
Right Ventricle	1 of 160	159 of 160	-24.01	-6.49	10.06
Skin	0 of 59	59 of 59	-12.68	1.5	22.77
Small Intestine	46 of 68	22 of 68	21.21	493.93	939.2
Soft Tissues	1 of 6	5 of 6	-1.99	2.6	5.32
Spleen	0 of 31	31 of 31	-9.41	-0.31	9.5
Stomach	7 of 47	40 of 47	19.02	52.29	117.09
Testis	0 of 5	5 of 5	-4.51	1.22	11.2
Thymus	1 of 71	70 of 71	-6.26	2.51	11.67
Thyroid Gland	1 of 18	17 of 18	-12.22	2.84	17.86
Uterus	0 of 58	58 of 58	-10.67	1.59	16.01
WBC	3 of 40	37 of 40	-16.45	-0.72	25.18

Shaded data corresponds to tissues wherein the highest levels of *NPC1L1* mRNA was detected. The "Present" column indicates the proportion of specified tissue samples evaluated wherein *NPC1L1* mRNA was detected. The "Absent" column indicates the proportion of specified tissue samples evaluated wherein *NPC1L1* RNA was not detected. The "lower 25%", "median" and "upper 75%" columns indicate statistical distribution of the relative NPC1L1 signal intensities observed for each set of tissue evaluated.

Example 8: Distribution of Rat NPC1L1, Rat IBAT or Rat SR-B1 mRNA in Rat Small Intestine.

In these experiments, the distribution of rat *NPC1L1* mRNA along the proximal-distal axis of rat small intestines was evaluated. Intestines were isolated from five independent animals and divided into 10 sections of approximately equal length. Total RNA was isolated and analyzed, by real time quantitative PCR using Taqman analysis, for localized expression levels of rat *NPC1L1*, rat *IBAT* (ileal bile acid transporter) or rat *SR-B1* mRNA. The primers and probes used in the analysis were:

rat *NPC1L1*:

Forward: TCTTCACCCTTGCTCTTTGC (SEQ ID NO: 23)

Reverse: AATGATGGAGAGTAGGTTGAGGAT (SEQ ID NO: 24)

Probe: [6FAM]TGCCCACCTTTGTTGTCTGCTACC[TAMRA] (SEQ ID NO: 25) rat Villin:

Forward: AGCACCTGTCCACTGAAGATTTC (SEQ ID NO: 26)

Reverse: TGGACGCTGAGCTTCAGTTCT (SEQ ID NO: 27)

Probe: [VIC]CTTCTCTGCGCTGCCTCGATGGAA[TAMRA] (SEQ ID NO: 28) rat *SR*-B1:

Forward: AGTAAAAAGGGCTCGCAGGAT (SEQ ID NO: 29)

Reverse: GGCAGCTGGTGACATCAGAGA (SEQ ID NO: 30)

Probe: [6FAM]AGGAGGCCATGCAGGCCTACTCTGA[TAMRA] (SEQ ID NO: 31) rat *IBAT*:

Forward: GAGTCCACGGTCAGTCCATGT (SEQ ID NO: 32)

Reverse: TTATGAACAACAATGCCAAGCAA (SEQ ID NO: 33)

Probe: [6FAM]AGTCCTTAGGTAGTGGCTTAGTCCCTGGAAGC TC[TAMRA] (SEQ ID NO: 34)

The mRNA expression levels of each animal intestinal section were analyzed separately, then the observed expression level was normalized to the observed level of villin mRNA in that intestinal section. The observed, normalized mRNA expression levels for each section where then averaged.

The expression level of *NPC1L1* and *SR-B1* were highest in the jejunum (sections 2-5) as compared to that of the more distal ileum sections. Since the jejunum is believed to be the site of cholesterol absorption, these data suggest such a role for rat NPC1L1. *IBAT* distribution favoring the ileum is well document and served as a control for the experiment.

Example 9: In situ Analysis of Rat NPC1L1 mRNA in Rat Jejunum Tissue.

The localization of rat *NPC1L1* mRNA was characterized by *in situ* hybridization analysis of rat jejunum serial sections. The probes used in this analysis were:

T7-sense probe: GTAATACGACTCACTATAGGGCCCTGACGGT CCTTCCTGAGGGAATCTTCAC (SEQ ID NO: 35)

T7-antisense probe: GTAATACGACTCACTATAGGGCCTGGGAA GTTGGTCATGGCCACTCCAGC (SEQ ID NO: 36)

The RNA probes were synthesized using T7 RNA polymerase amplification of a PCR amplified DNA fragment corresponding rat *NPC1L1* nucleotides 3318 to 3672 (SEQ ID NO 1). Sense and anti-sense digoxigenin–UTP labeled cRNA probes were generated from the T7 promoter using the DIG RNA Labeling Kit following the manufacturer's instructions. Serial cryosections rat jejunum were hybridized with the sense and antiisense probes. Digoxigenin labeling was detected with the DIG Nucleic Acid Detection Kit based on previous methods. A positive signal is characterized by the deposition of a red reaction product at the site of hybridization.

The anti-sense probe showed strong staining of epithelium along the crypt-villus axis under low magnification (40X). The observed rat *NPC1L1* mRNA expression levels may have been somewhat greater in the crypts than in the villus tips. Under high magnification (200X), staining was observed in the enterocytes but not in the goblet cells. A lack of staining observed with the sense probe (control) confirmed the high specificity of the *NPC1L1* anti-sense signal. These data provided further evidence of the role of rat NPC1L1 in intestinal cholesterol absorption.

Example 10: FACS Analysis of Fluorescently Labeled Ezetimibe Binding to Transiently Transfected CHO Cells.

In these experiments, the ability of BODIPY-labeled ezetimibe (Altmann, *et al.*, (2002) Biochim. Biophys. Acta 1580(1): 77-93) to bind to *NPC1L1* and *SR-B1* was evaluated. "BODIPY" is a fluorescent group which was used to detect the BODIPY-ezetimibe. Chinese hamster ovary (CHO) cells were transiently transfected with rat *NPC1L1* DNA (rNPC1L1/CHO), mouse *NPC1L1* DNA (mNPC1L1/CHO), mouse *SR-B1* DNA (mSRBI/CHO) or *EGFP* DNA (EGFP/CHO). EGFP is enhanced green fluorescent protein which was used as a positive control. The transfected CHO cells or untransfected CHO cells were then stained with 100 nM BODIPY-labeled ezetimibe and analyzed by FACS. Control experiments were also performed wherein the cells were not labeled with the BODIPY-ezetimibe and wherein untransfected CHO cells were labeled with the BODIPY-ezetimibe.

No staining was observed in the untransfected CHO, rNPC1L1/CHO or mNPC1L1/CHO cells. Fluorescence was detected in the positive-control

EGFP/CHO cells. Staining was also detected in the mouse SR-B1/CHO cells. These data show that, under the conditions tested, BODIPY-ezetimibe is capable of binding to SR-B1 and that such binding is not ablated by the presence of the fluorescent BODIPY group. When more optimal conditions are determined, BODIPY-ezetimibe will be shown to label the rNPC1L1/CHO and mNPC1L1/CHO cells.

Example 11: FACS Analysis of Transiently Transfected CHO Cells Labeled with Anti-FLAG Antibody M2.

In these experiments, the expression of FLAG-tagged NPC1L1 on CHO cells was evaluated. CHO cells were transiently transfected with mouse *NPC1L1* DNA, rat *NPC1L1* DNA, FLAG- rat *NPC1L1* DNA or FLAG- mouse *NPC1L1* DNA. The 8 amino acid FLAG tag used was DYKDDDDK (SEQ ID NO: 37) which was inserted on the amino-terminal extracellular loop just past the secretion signal sequence. The cells were incubated with commercially available anti-FLAG monoclonal mouse antibody M2 followed by a BODIPY-tagged anti-mouse secondary antibody. The treated cells were then analyzed by FACS.

The M2 antibody stained the CHO cells transfected with FLAG-rat *NPC1L1* DNA and with FLAG–mouse NPC1L1. No staining was observed in the CHO cells transfected with mouse *NPC1L1* DNA and with rat *NPC1L1* DNA. These data showed that rat NPC1L1 and mouse NPC1L1 possess no significant, inherent fluorescence and are not bound by the anti-FLAG antibody. The observed, FLAG-dependent labeling of the cells indicated that the FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 proteins are localized at the cell membrane of the CHO cells.

Example 12: FACS Analysis of FLAG-rat NPC1L1-EGFP Chimera in Transiently Transfected CHO Cells.

In these experiments, the surface and cytoplasmic localization of rat NPC1L1 in CHO cells was evaluated. CHO cells were transiently transfected with FLAG- rat *NPC1L1* DNA or with FLAG-rat *NPC1L1-EGFP* DNA. In these fusions, the FLAG tag is at amino-terminus of rat NPC1L1 and EGFP fusion is at the carboxy-terminus of rat NPC1L1. The cells were then stained with the M2 anti-FLAG mouse (primary) antibody followed by secondary staining with a BODIPY-labeled anti-mouse antibody. In control experiments, cells were stained with only the secondary antibody and not with the primary antibody (M2). The stained cells were then analyzed by FACS.

In a control experiment, FLAG-rat NPC1L1 transfected cells were stained with BODIPY anti-mouse secondary antibody but not with the primary antibody. The data demonstrated that the secondary, anti-mouse antibody possessed no significant specificity for FLAG-rat NPC1L1 and that the FLAG-rat NPC1L1, itself, possesses no significant fluorescence.

In another control experiment, unlabeled FLAG-rat NPC1L1-EGFP cells were FACS analyzed. In these experiments, autofluorescence of the enhanced green fluorescent protein (EGFP) was detected.

FLAG-rat NPC1L1 cells were stained with anti-FLAG mouse antibody M2 and with the BODIPY-labeled anti-mouse secondary antibody and FACS analyzed. The data from this analysis showed that the cells were labeled with the secondary, BODIPY-labeled antibody which indicated expression of the FLAG-rat NPC1L1 protein on the surface of the CHO cells.

FLAG-rat NPC1L1-EGFP cells were stained with anti-FLAG mouse antibody M2 and with the BODIPY-labeled anti-mouse secondary antibody and FACS analyzed. The data from this analysis showed that both markers (BODIPY and EGFP) were present indicating surface expression of the chimeric protein. The data also indicated that a portion of the protein was located within the cells and may be associated with transport vesicles. These data supported a role for rat NPC1L1 in vesicular transport of cholesterol or protein expressed in subcellular organelles such as the rough endoplasmic reticulum.

Example 13: FACS Analysis and Fluorescent Microscopy of FLAG-rat NPC1L1-EGFP Chimera in a Cloned CHO Cell Line.

In these experiments, the cellular localization of rat NPC1L1 was evaluated by FACS analysis and by immunohistochemistry. CHO cells were transfected with FLAG-rat *NPC1L1-EGFP* DNA and stained with anti-FLAG mouse antibody M2 and then with a BODIPY-labeled anti-mouse secondary antibody. In the fusion, the FLAG tag is at the amino-terminus of rat NPC1L1 and the enhanced green fluorescent protein (EGFP) tag is located at the carboxy-terminus of the rat NPC1L1. The stained cells were then analyzed by FACS and by fluorescence microscopy.

Cells transfected with FLAG—rat *NPC1L1-EGFP* DNA were stained with the anti-FLAG mouse antibody M2 and then with the BODIPY-labeled anti-

mouse secondary antibody. FACS analysis of the cells detected both markers indicating surface expression of the chimeric protein.

FLAG–rat NPC1L1-EGFP transfected cells were analyzed by fluorescent microscopy at 63X magnification. Fluorescent microscopic analysis of the cells indicated non-nuclear staining with significant perinuclear organelle staining. Resolution of the image could not confirm the presence of vesicular associated protein. These data indicated that the fusion protein was expressed on the cell membrane of CHO cells.

Example 14: Generation of Polyclonal Anti-rat NPC1L1 Rabbit Antibodies.

Synthetic peptides (SEQ ID NO: 39-42) containing an amino- or carboxy-terminal cysteine residue were coupled to keyhole limpet hemocyanin (KLH) carrier protein through a disulfide linkage and used as antigen to raise polyclonal antiserum in New Zealand white rabbits (range 3-9 months in age). The KLH-peptide was emulsified by mixing with an equal volume of Freund's Adjuvant, and injected into three subcutaneous dorsal sites. Prior to the 16 week immunization schedule a pre-immune sera sample was collected which was followed by a primary injection of 0.25 mg KLH-peptide and 3 scheduled booster injections of 0.1 mg KLH-peptide. Animals were bled from the auricular artery and the blood was allowed to clot and the serum was then collected by centrifugation

The anti-peptide antibody titer was determined with an enzyme linked immunosorbent assay (ELISA) with free peptide bound in solid phase (1μg/well). Results are expressed as the reciprocal of the serum dilution that resulted in an OD_{450} of 0.2. Detection was obtained using the biotinylated anti-rabbit IgG, horse radish peroxidase–streptavidin (HRP-SA) conjugate, and ABTS.

Example 15: FACS Analysis of Rat NPC1L1 Expression in CHO Cells Transiently Transfected with Rat NPC1L1 DNA Using Rabbit Anti-rat NPC1L1 Antisera.

In these experiments, the expression of rat NPC1L1 on the surface of CHO cells was evaluated. CHO cells were transfected with rat *NPC1L1* DNA, then incubated with either rabbit preimmune serum or with 10 week anti-rat NPC1L1 serum described, above, in Example 14 (i.e., A0715, A0716, A0867 or A0868). Cells labeled with primary antisera were then stained with a BODIPY-modified anti-rabbit secondary antibody followed by FACS analysis.

No antibody surface labeling was observed for any of the pre-immune sera samples. Specific cell surface labeling of rat NPC1L1 transfected cells was observed for both A0715 and A0868. Antisera A0716 and A0867 did not recognize rat NPC1L1 surface expression in this assay format. This indicates that the native, unfused rat NPC1L1 protein is expressed in the CHO cells and localized to the CHO cell membranes. Cell surface expression of NPC1L1 is consistent with a role in intestinal cholesterol absorption.

Example 16: FACS Analysis of CHO Cells Transiently Transfected with FLAG-Mouse NPC1L1 DNA or FLAG-rat NPC1L1 DNA or Untransfected CHO Cells Using Rabbit Anti-rat NPC1L1 Antisera.

In these experiments, the expression of FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 in CHO cells was evaluated. CHO cells were transiently transfected with FLAG-mouse *NPC1L1* DNA or with FLAG-rat *NPC1L1* DNA. The FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 transfected cells were labeled with either A0801, A0802, A0715 or A0868 sera (see Example 14) or with anti-FLAG antibody, M2. The labeled cells were then stained with BODIPY-labeled anti-rabbit secondary antibody and FACS analyzed. The untransfected CHO cells were analyzed in the same manner as the transfected cell lines.

Positive staining of the untransfected CHO cells was not observed for any of the antisera tested. Serum A0801-dependent labeling of FLAG-rat NPC1L1 transfected cells was observed but such labeling of FLAG-mouse NPC1L1 transfected cells was not observed. Serum A0802-dependent labeling of FLAG-mouse NPC1L1 or FLAG-rat NPC1L1 transfected cells was not observed. Strong serum A0715-dependent labeling of FLAG-rat NPC1L1 transfected cells was observed and weak serum A0715-dependent labeling of FLAG-mouse NPC1L1 transfected cells was observed. Weak serum A0868-dependent labeling of rat NPC1L1 and mouse NPC1L1 transfected cells was observed. Strong Anti-FLAG M2 antibody-dependent labeling of FLAG-rat NPC1L1 and FLAG-mouse NPC1L1 transfected cells was observed. The strong M2 staining is likely to be due to the fact that M2 is an affinity-purified, monoclonal antibody of known concentration. In contrast, the respective antisera are polyclonal, unpurified and contain an uncertain concentration of anti-rat NPC1L1 antibody. These date provide further evidence that the FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 proteins are expressed in CHO cells and localized to

the CHO cell membranes. Cell surface expression of NPC1L1 is consistent with a role in intestinal cholesterol absorption.

Example 17: Immunohistochemical Analysis of Rat Jejunum Tissue with Rabbit Anti-rat NPC1L1 Antisera A0715.

In these experiments, the localization of rat NPC1L1 in rat jejunum was analyzed by immunohistochemistry. Rat jejunum was removed, immediately embedded in O.C.T. compound and frozen in liquid nitrogen. Sections (6μm) were cut with a cryostat microtome and mounted on glass slides. Sections were air dried at room temperature and then fixed in Bouin's fixative. Streptavidin-biotin-peroxidase immunostaining was carried out using Histostain-SP kit. Endogenous tissue peroxidase activity was blocked with a 10 minute incubation in 3% H_2O_2 in methanol, and nonspecific antibody binding was minimized by a 45 minute incubation in 10% nonimmune rabbit serum. Sections were incubated with a rabbit anti-rat NPC1L1 antisera A0715 or A0868 at a 1: 500 dilution at 4°C, followed by incubation with biotinylated goat anti-rabbit IgG and with streptavidin-peroxidase. Subsequently, the sections were developed in an aminoethyl carbazole (AEC)-H_2O_2 staining system and counterstained with hematoxylin and examined by microscopy. A positive reaction using this protocol is characterized by the deposition of a red reaction product at the site of the antigen-antibody reaction. Nuclei appeared blue from the hematoxylin counterstain. Controls were performed simultaneously on the neighboring sections from the same tissue block. Control procedures consisted of the following: (1) substitute the primary antibody with the pre-immune serum, (2) substitute the primary antibody with the non-immune rabbit serum, (3) substitute the primary antibody with PBS, (4) substitute the second antibody with PBS.

The example shows tissue stained with anti-rat NPC1L1 sera A0715 or with the preimmune sera analyzed at low magnification (40X) and at high magnification (200X). The A0715-stained tissue, at low magnification, showed positive, strong staining of the villi epithelial layer (enterocytes). The A0715-stained tissue at high magnification showed positive, strong staining of the enterocyte apical membranes. No staining was observed in tissue treated only with preimmune sera. Similar results were obtained with sera A0868. These data indicate that rat NPC1L1 is expressed in rat jejunum which is consistent with a role in intestinal cholesterol absorption.

Example 18: Labeled Cholesterol Uptake Assay.

In this example, the ability of CHO cells stably transfected with rat NPC1L1 to take up labeled cholesterol was evaluated. In these assays, cholesterol uptake, at a single concentration, was evaluated in a pulse-chase experiment. The data generated in these experiments are set forth, below, in Table 3.

Cells:

A. CHO cells stably transfected with rat *NPC1L1* cDNA

B. CHO background (no transfection)

Cells were seeded at 500,000 cells/ well (mL) in 12-well plates.

Procedure:

All reagents and culture plates were maintained at 37°C unless otherwise noted.

Starve. The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed and the cells were rinsed with serum-free HAMS media. The serum-free media was then replaced with 1 mL "starve" media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS).

One plate of each cell line was starved overnight. The remaining 2 plates were designated "No Starve" (see below).

Pre-Incubation. Media was removed from all plates, rinsed with serum-free HAMS and replaced with starve media for 30 minutes.

^{3}H-Cholesterol Pulse. The following was added directly to each well.

0.5μCi ^{3}H-cholesterol (~1.1 X 10^{6} dpm/well) in 50μl of a mixed bile salt micelle.

4.8 mM sodium taurocholate (2.581 mg/mL)

0.6 mM sodium oleate (0.183 mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

Dispersed in "starve" media by ultrasonic vibration

Final media cholesterol concentration = 5μg/mL

Labeled cholesterol pulse time points were 0, 4, 12 and 24 minutes. Triplicate wells for each treatment were prepared.

Wash. At the designated times, media was aspirated and the cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% BSA, pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

Processing/Analysis. Cells were digested overnight with 0.2N NaOH, 2mL/well at room temperature. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting. Two additional 50µl aliquots from all wells are assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized by the quantity of protein in the cells.

Table 3. Uptake of 3H-cholesterol by CHO cells transfected with rat *NPC1L1* or mouse *SR-B1* or untransfected CHO cells.

Time, min After ^{3}H-Cholesterol	Total Cholesterol, dpm protein ± sem				Total Cholesterol, dpm/mg protein ± sem			
	NPC1L1		CHO		NPC1L1		CHO	
	No Starve							
0	2067	±46	4568	±1937	10754	±166	22881	±9230
4	2619	±130	2868	±193	15366	±938	15636	±1471
12	2868	±193	4459	±170	15636	±1471	24622	±966
24	7010	±89	7204	±173	41129	±685	39361	±1207
	Starve							
0	1937	±273	2440	±299	10909	±1847	12429	±1673
4	3023	±308	2759	±105	17278	±1650	14307	±781
12	2759	±105	4857	±186	14307	±781	26270	±1473
24	6966	±72	7344	±65	39196	±174	38381	±161

dpm=disintegrations per minute
sem=standard error of the mean

Example 19: Effect of Ezetimibe on Cholesterol Uptake.

The effect of ezetimibe on the ability of CHO cells stably transfected with mouse or rat *NPC1L1* or mouse *SR-B1* to take up ^{3}H-labeled cholesterol was evaluated in pulse-chase experiments. One cDNA clone of mouse *NPC1L1* (C7) and three clones of rat *NPC1L1* (C7, C17 and C21) were evaluated. The ability of CHO

cells stably transfected with mouse *SR-B1*, mouse *NPC1L1* and rat *NPC1L1* to take up labeled cholesterol, in the absence of ezetimibe, was also evaluated in the pulse-chase experiments. Data generated in these experiments are set forth, below, in Tables 4 and 5. Additionally, the quantity of total cholesterol taken up by transfected and untransfected CHO cells in the presence of four different unlabeled cholesterol concentrations was also evaluated. The data from these experiments is set forth, below, in Table 6.

Cells:

A. CHO cells stably transfected with rat or mouse *NPC1L1* cDNA

B. CHO background (no transfection)

C. *SR-B1* transfected CHO cells

Cells seeded at 500,000 cells / well (mL) in 12-well plates.

Procedure:

All reagents and culture plates were maintained at 37°C unless otherwise noted.

Starve. The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed and the cells were rinsed with serum-free HAMS media. The serum-free media was then replaced with 1 mL "starve" media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS). The cells were then starved overnight.

Pre-Incubation/ pre-dose. Media was removed from all plates and replaced with fresh starve media and preincubated for 30 minutes. Half of the wells received media containing ezetimibe (stock soln in EtOH; final conc. = 10μM).

^{3}H-Cholesterol Pulse. The following was added directly to each well:

0.5μCi ^{3}H-cholesterol (~1.1 X 106 dpm/well) in 50μl of a mixed bile salt micelle

4.8mM sodium taurocholate (2.581mg/mL)

0.6 mM sodium oleate (0.183 mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

Dispersed in "starve" media by ultrasonic vibration

Final media cholesterol concentration = 5μg/mL

Labeled cholesterol pulse time points were 4, 12, 24 minutes and 4 hours. Triplicate wells were prepared for each treatment.

Wash. At designated times, media was aspirated and cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% bovine serum albumin (BSA), pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

Processing/Analysis.

A. 4, 12, 24 minute time points: Cells were digested overnight with 0.2N NaOH, 2mL/well, room temperature. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting.

B. 4 hour time point: The digested cells were analyzed by thin-layer chromatography to determine the content of cholesterol ester in the cells.

Extracts were spotted onto TLC plates and run for 30 minutes in 2 ml hexane: isopropanol (3: 2) mobile phase for 30 minutes, followed by a second run in 1 ml hexane: isopropanol (3: 2) mobile phase for 15 minutes.

C. Protein determination of cell extracts. Plates containing a sample of the cell extracts were placed on orbital shaker at 120 rpm for indicated times and then extracts are pooled into 12 X 75 tubes. Plates were dried and NaOH (2ml/well) added. The protein content of the samples were then determined. Two additional 50µl aliquots from all wells were assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized to the quantity of protein in the cells.

Table 4. Total Cholesterol in Transfected CHO Cells in the Presence and Absence of

Ezetimibe.

Clones:	Total Cholesterol, dpm ± sem				Total Cholesterol, dpm/mg protein ± sem			
	Vehicle		EZ (10 μM)		Vehicle		EZ (10μM)	
	4 Min Pulse							
CHO Control	3413	±417	3222	±26	33443	±4070	31881	±483
SR-BI	14207	±51	10968	±821	118242	±1261	92474	±2902
mNPC1L1(C7)	4043	±419	4569	±222	30169	±3242	30916	±1137
rNPC1L1(C21)	3283	±288	3769	±147	23728	±2111	27098	±689
rNPC1L1(C17)	3188	±232	3676	±134	24000	±832	28675	±527
rNPC1L1(C7)	1825	±806	3268	±121	15069	±6794	27285	±968
	12 Min Pulse							
CHO Control	4710	±246	4532	±165	44208	±2702	43391	±1197
SR-BI	16970	±763	12349	±298	140105	±6523	98956	±4447
mNPC1L1(C7)	6316	±85	6120	±755	45133	±342	41712	±4054
rNPC1L1(C21)	5340	±12	4703	±231	40018	±1181	33985	±1928
rNPC1L1(C17)	4831	±431	4579	±257	37378	±3461	34063	±1619
rNPC1L1(C7)	4726	±272	4664	±63	39100	±2350	38581	±784
	24 Min Pulse							
CHO Control	7367	±232	6678	±215	65843	±1281	61764	±2131
SR-BI	39166	±2152	23558	±1310	324126	±11848	198725	±11713
mNPC1L1(C7)	10616	±121	9749	±482	77222	±1040	74041	±3670
rNPC1L1(C21)	9940	±587	8760	±293	76356	±9618	66165	±2181
rNPC1L1(C17)	8728	±721	8192	±237	70509	±5189	62279	±4352
rNPC1L1(C7)	8537	±148	7829	±204	72134	±1305	63482	±368

EZ = ezetimibe

Table 5. Cholesterol Ester in CHO cells in the Presence or Absence of Ezetimibe.

	Cholesteryl Ester, dpm ± sem				Cholesteryl Ester, dpm/mg protein ± sem			
	Vehicle		EZ (10 μM)		Vehicle		EZ (10μM)	
Clones:	4 Hour Pulse							
CHO Control	652	±13	208	±9	5647	±55	1902	±87
SR-BI	47608	±1292	9305	±401	391067	±14391	72782	±3181
mNPC1L1(C7)	732	±127	453	±118	4994	±827	3057	±776
rNPC1L1(C21)	2667	±90	454	±33	18655	±1032	3193	±265
rNPC1L1(C17)	751	±74	202	±10	5379	±481	1510	±62
rNPC1L1(C7)	462	±25	191	±54	3597	±193	1496	±403

	Free Cholesterol, dpm ± sem				Free Cholesterol, dpm/mg protein ± sem			
	Vehicle		EZ (10 μM)		Vehicle		EZ (10μM)	
	4 Hour Pulse							
CHO Control	61612	±1227	56792	±568	533876	±17770	519607	±16203
SR-BI	214678	±4241	194519	±474	1762873	±46607	1521341	±4185
mNPC1L1(C7)	79628	±793	77516	±1910	544661	±1269	523803	±10386
rNPC1L1(C21)	71352	±1343	69106	±711	498016	±8171	485460	±4410
rNPC1L1(C17)	78956	±3782	71646	±446	566456	±29204	536651	±7146
rNPC1L1(C7)	75348	±2093	70628	±212	586127	±13932	556855	±7481

EZ =ezetimibe

Table 6. Uptake of labeled cholesterol in the presence of increasing amounts of unlabeled cholesterol.

Cold Cholesterol	Total Cholesterol, dpm ± sem				Total Cholesterol, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
	24 Min Pulse							
3 µg/mL	12271 ±430	49603 ±2428	14250 ±1628	10656 ±1233	108936 ±5413	541562 ±13785	140764 ±14433	94945 ±12916
10 µg/mL	16282 ±2438	79967 ±8151	25465 ±3037	13225 ±4556	151283 ±23345	880224 ±82254	250985 ±27481	123433 ±34092
30 µg/mL	14758 ±1607	71925 ±3863	19001 ±1530	13218 ±1149	135109 ±12106	796236 ±18952	180436 ±12112	111522 ±6941
100 µg/mL	16458 ±1614	58185 ±4548	15973 ±1665	11560 ±1132	149559 ±17977	630143 ±3718	147717 ±8261	101328 ±7191
	Cholesteryl Ester, dpm ± sem				Cholesteryl Ester, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
	4 Hour Pulse							
3 µg/mL	2737 ±114	39596 ±1241	1561 ±1	4015 ±47	22050 ±978	382641 ±5955	13684 ±217	32020 ±641
10 µg/mL	1646 ±76	17292 ±362	998 ±36	1866 ±33	13323 ±606	157914 ±3400	8917 ±467	14849 ±127
30 µg/mL	970 ±46	6642 ±153	537 ±82	970 ±9	7627 ±325	63547 ±1760	4885 ±748	7741 ±100
100 µg/mL	895 ±156	4777 ±27	405 ±7	777 ±16	7135 ±1230	45088 ±1526	3663 ±68	6005 ±198
	Free Cholesterol, dpm ± sem				Free Cholesterol, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
	4 Hour Pulse							
3 µg/mL	89013 ±3724	211783 ±3268	104343 ±2112	92244 ±987	717308 ±34130	2047695 ±16213	914107 ±5869	735498 ±11209
10 µg/mL	136396 ±8566	278216 ±10901	196173 ±4721	125144 ±877	1105118 ±76074	2540130 ±92471	1753072 ±86578	996824 ±27850
30 µg/mL	131745 ±2922	224429 ±2556	149172 ±19689	117143 ±4976	1036195 ±21142	2149315 ±78068	1357136 ±180264	934772 ±43202
100 µg/mL	79336 ±4011	231470 ±4221	114599 ±2803	93538 ±1588	632965 ±29756	2182022 ±36793	1035979 ±30329	723225 ±21694
	Cholesteryl Ester, dpm ± sem				Cholesteryl Ester, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
	24 Hour Pulse							
3 µg/mL	57373 ±2704	162296 ±1644	22986 ±940	59377 ±953	357629 ±14639	1248900 ±18565	160328 ±6565	401315 ±5557
10 µg/mL	33730 ±1296	112815 ±373	14836 ±552	31797 ±525	215004 ±5942	830231 ±12764	98594 ±4205	200451 ±5239
30 µg/mL	19193 ±100	58668 ±1413	8878 ±355	18963 ±380	122071 ±1271	446581 ±3472	59091 ±2697	119728 ±2131
100 µg/mL	16761 ±398	31280 ±1270	8784 ±946	14933 ±311	103235 ±1739	272796 ±13392	60670 ±4597	96215 ±1023
	Free Cholesterol, dpm ± sem				Free Cholesterol, dpm/mg protein ± sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
	24 Hour Pulse							
3 µg/mL	248985 ±4207	357819 ±4519	285610 ±5187	227244 ±1016	1552637 ±18954	2752957 ±24984	1993256 ±56968	1536023 ±10304
10 µg/mL	231208 ±8927	269822 ±5872	311777 ±8227	231666 ±6198	1477414 ±85954	1984473 ±18420	2069980 ±25517	1461157 ±58517
30 µg/mL	203566 ±6008	225273 ±5932	279604 ±6612	209372 ±3386	1294878 ±41819	1716066 ±52581	1859476 ±29507	1321730 ±5452
100 µg/mL	178424 ±2379	167082 ±2211	229832 ±4199	182678 ±7709	1099648 ±25160	1455799 ±9885	1599244 ±76938	1177546 ±51191

Example 20: Labeled Cholesterol Uptake Assay.

In this example, the ability of CHO cells transiently transfected with rat *NPC1L1* or mouse *SR-B1* to take up labeled cholesterol was evaluated. Also evaluated was the ability of rat *NPC1L1* to potentiate the ability of CHO cells transfected with mouse *SR-B1* to take up labeled cholesterol. In these assays,

cholesterol uptake, at a single concentration, was evaluated in pulse-chase experiments. The data generated in these experiments are set forth, below, in Table 7.

Cells:

A. CHO background cells (mock transfection).

B. CHO cells transiently transfected with mouse SR-B1.

C. CHO transiently transfected with rat *NPC1L1* cDNAs (n=8 clones).

Transiently transfected cells were seeded at 300,000 cells / well (mL) in 12-well plates.

Procedure:

All reagents and culture plates were maintained at 37°C unless otherwise noted.

Starve. The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed from the cells and replaced with 1 mL "starve" media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS). Cells were starved for 1 hour.

^{3}H-Cholesterol Pulse. The following was added directly to each well.

0.5μCi ^{3}H-cholesterol (~1.1 X 106 dpm/well) in 50μl of a mixed bile salt micelle.

4.8mM sodium taurocholate (2.581mg/mL)

0.6 mM sodium oleate (0.183 mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

Dispersed in "starve" media by ultrasonic vibration

Final media cholesterol concentration = 5μg/mL

Labeled cholesterol pulse time points were 24 Min and 4 hours.

Triplicate wells for each treatment.

Wash. At the designated times, media was aspirated and cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% BSA, pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

Processing/Analysis.

A. 24 minute time point: Cells were digested overnight with 0.2N NaOH, 2mL/well at room temp. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting.

B. 4 hour time point: The digested cells were analyzed by thin-layer chromatography to determine the content of cholesterol ester in the cells.

The extracts were spotted onto thin layer chromatography plates and run in 2 ml hexane: isopropanol (3: 2) containing mobile phase for 30 minutes, followed by a second run in 1 ml hexane: isopropanol (3: 2) containing mobile phase for 15 min.

C. Protein determination of cell extracts: Plates containing a sample of the cell extracts were placed on orbital shaker at 120 rpm for indicated times and then extracts are pooled into 12X75 tubes. Plates were dried and NaOH (2ml/well) added. The protein content of the samples were then determined. Two additional 50µl aliquots from all wells were assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized to the quantity of protein in the cells.

Table 7. Labeled cholesterol uptake in transiently transfected CHO cells.

	Total Cholesterol, ± sem	
	dpm	dpm/mg protein
Transfection	**24 Min Pulse**	
CHO Control (mock)	4721 ±436	49024 ±4328
SR-BI(Transient)	5842 ±82	59445 ±1099
NPC1L1 (Transient)	4092 ±377	47026 ±2658
SR-BI/NPC1L1 (trans)	3833 ±158	52132 ±3071
	Cholesteryl Ester, ± sem	
	dpm	**dpm/mg protein**
	4 Hour Pulse	
CHO Control (mock)	2132 ±40	20497 ±640
SR-BI(Transient)	5918 ±237	51812 ±1417
NPC1L1 (Transient)	1944 ±93	19788 ±642
SR-BI/NPC1L1 (trans)	4747 ±39	58603 ±1156
	Free Cholesterol, ± sem	
	dpm	**dpm/mg protein**
	4 Hour Pulse	
CHO Control (mock)	45729 ±328	439346 ±5389
SR-BI(Transient)	50820 ±2369	444551 ±9785
NPC1L1 (Transient)	39913 ±1211	406615 ±6820
SR-BI/NPC1L1 (trans)	37269 ±1225	459509 ±6195

Example 21: Expression of rat, mouse and human NPC1L1.

In this example, *NPC1L1* was introduced into cells and expressed. Species specific NPC1L1 expression constructs were cloned into the plasmid pCDNA3 using clone specific PCR primers to generate the ORF flanked by appropriate restriction sites compatible with the polylinker of the vector. For all three species of NPC1L1, small intestine total tissue RNA was used as a template for reverse transcriptase-polymerase chain reaction (RT-PCR) using oligo dT as the

template primer. The rat *NPC1L1* was cloned as an EcoRI fragment, human *NPC1L1* was cloned as a XbaI/NotI fragment and mouse *NPC1L1* was cloned as an EcoRI fragment. Forward and reverse strand sequencing of each clone was performed to confirm sequence integrity. Standard transient transfection procedures were used with CHO cells. In a 6-well plate CHO cells were plated 1 day before transfection at a plating density of 2 X 10^5 cells/well. The following day, cells were incubated with 2 μg plasmid DNA and 6 μL Lipofectamine for 5 hours followed a fresh media change. Forty-eight hours later, cells were analyzed for NPC1L1 expression using anti-NPC1L1 antisera by either FACS or western blot. To establish stable long term cell lines expressing NPC1L1, transfected CHO cells were selected in the presence of geneticin (G418, 0.8 mg/ml) as recommended by the manufacturer (Life Technologies). Following one month of selection in culture, the cell population was stained with anti-NPC1L1 antisera and sorted by FACS. Individual positive staining cells were cloned after isolation by limiting dilution and then maintained in selective media containing geneticin (0.5 mg/ ml).

Other cell types less susceptible to transfection procedures have been generated using adenoviral vector systems. This system used to express NPC1L1 is derived from Ad 5, a type C adenovirus. This recombinant replication-defective adenoviral vector is made defective through modifications of the E1, E2 and E4 regions. The vector also has additional modifications to the E3 region generally affecting the E3b region genes RIDa and RIDb. NPC1L1 expression was driven using the CMV promoter as an expression cassette substituted in the E3 region of the adenovirus. Rat and mouse NPC1L1 were amplified using clone specific primers flanked by restriction sites compatible with the adenovirus vector Adenovirus infective particles were produced from 293-D22 cells in titers of 5 X 10^{10} P/mL. Viral lysates were used to infect cells resistant to standard transfection methodologies. In Caco2 cells, which are highly resistant to heterologous protein expression, adenovirus mediated expression of NPC1L1 has been shown by western blot analysis to persist at least 21 days post-infection.

Example 22: NPC1L1 Knock-Out Transgenic Mouse.

NPC1L1 knockout mice were constructed via targeted mutagenesis. This methodology utilized a targeting construct designed to delete a specific region of the mouse *NPC1L1* gene. During the targeting process the *E. coli* lacZ reporter gene

was inserted under the control of the endogenous *NPC1L1* promoter. The region in *NPC1L1* (SEQ ID NO: 45) being deleted is from nucleotide 790 to nucleotide 998. The targeting vector contains the LacZ-Neo cassette flanked by 1.9 kb 5' arm ending with nucleotide 789 and a 3.2 kb 3' arm starting with nucleotide 999. Genomic DNA from the recombinant embryonic stem cell line was assayed for homologous recombination using PCR. Amplified DNA fragments were visualized by agarose gel electrophoresis. The test PCRs employed a gene specific primer, which lies outside of and adjacent to the targeting vector arm, paired with one of three primers specific to the Lac*Z-Neo* cassette sequence. For 5' PCR reconfirmation, the *NPC1L1* specific oligonucleotide ATGTTAGGTGAGTCTGAACCTACCC (SEQ ID NO: 46) and for 3'PCR reconfirmation the NPC1L1 specific oligonucleotide GGATTGCATTTCCTTCAAGAAAGCC (SEQ ID NO: 47) were used. Genotyping of the F2 mice was performed by multiplex PCR using the NPC1L1 specific forward primer TATGGCTCTGCCCTCTGCAATGCTC (SEQ ID NO: 48) the LacZ-Neo cassette specific forward primer TCAGCAGCCTCTGTTCCACATACACTTC (SEQ ID NO: 49) in combination with the NPC1L1 gene specific reverse primer GTTCCACAGGGTCTGTGGTGAGTTC (SEQ ID NO: 50) allowed for determination of both the targeted and endogenous alleles. Analysis of the PCR products by agarose gel electrophoresis distinguished the wild-type, heterozygote and homozygote null mouse from each other.

Example 23: Acute Cholesterol Absorption in NPC1L1-Deficient Mice.

To determine whether NPC1L1 plays a role in cholesterol absorption, *NPC1L1* deficient mice were studied.

Mice deficient in *NPC1L1* (-/-) were generated by breeding heterozygote mice (+/) to obtain wild-type (+/+) and *NPC1L1* deficient mice (-/-). Non-fasted mice (6.5-9 weeks old, mixed 129 and C57BL/6 background) were weighed and grouped (n=2 -/- and n=4 +/+). All animals were gavaged (Feeding needles, 24G x 1 inch, Popper and Sons, NY) with 0.1 ml corn oil (Sigma; St. Louis, MO) containing 1μCi ^{14}C-cholesterol (New England Nuclear, [$^{4\text{-}14}$C] Cholesterol, NEC-018) and 0.1mg carrier cholesterol mass (Sigma; St. Louis, MO). Two hours later, blood was collected by heart puncture. The liver was removed, weighed, and three samples were placed into 20 ml counting vials. Tissues were digested in 1 ml of 1N NaOH at 60°C overnight. The tissue digests were acidified by addition of 250μl

of 4N HCl prior to liquid scintillation counting (LSC). Plasma was isolated by centrifugation at 10,000 rpm for 5 minutes in a microfuge and duplicate 100µl aliquots of plasma were taken for LSC.

Cholesterol absorption, evaluated by this acute technique and expressed as the total amount of radioactive cholesterol in the plasma and liver, demonstrated that the wild type mice (+/+) absorbed an average of 11,773 dpm and *NPC1L1* deficient mice absorbed 992 dpm of the 14C-cholesterol. These results indicate that the *NPC1L1* deficient mice have a 92% reduction in cholesterol absorption. These data confirm the role of NPC1L1 in intestinal cholesterol absorption. Inhibition of NPC1L1-mediated cholesterol absorption, in a subject, by administering NPC1L1 antagonists, such as ezetimibe, to the subject, are a useful way to reduce serum cholesterol levels and the occurrence of atherosclerosis in the subject.

Example 24: Cholesterol Absorption in NPC1L1 (NPC3) Knockout Mice (Fecal Ratio Method: Cholesterol/Sitostanol).

In this example, cholesterol absorption and the activity of ezetimibe was determined in the *NPC1L1* knockout mice (-/-), heterozygous mice (+/-), and age matched wild-type mice (+/+).

Cholesterol absorption in the mice was determined by the dual fecal isotope ratio method as described by Altmann *et al.* (Biochim. Biophys. Acta. 1580(1): 77-93 (2002)). Mice (n= 4-6/group) were fed a standard rodent chow diet and in some groups treated daily with a maximally effective dose of ezetimibe (10 mg/kg). Mice were gavaged with ^{14}C-cholesterol (1µCi, 0.1mg unlabeled cholesterol) and ^{3}H-sitostanol (2µCi) in 0.1ml corn oil. Feces were collected for 2 days and fecal ^{14}C-cholesterol and ^{3}H-sitostanol levels were determined by combustion in a Packard Oxidizer. The fraction of cholesterol absorbed, as evaluated by the fecal dual isotope technique, was similar in wild type (+/+) and heterozygous mice (+/-) fed a chow diet (heterozygous mice absorbed 46 ±5% and age matched wild type mice absorbed 51 ±3% of the dose of ^{14}C-cholesterol). The *NPC1L1* knockout mice (-/-) absorbed 15.6 ±0.4% of the ^{14}C-cholesterol, which was similar to the wild type mice treated with a maximally effective dose of ezetimibe (16.1 ±0.3%), and reduced by 69% compared to wild type mice ($p<0.001$). In *NPC1L1* knockout treated with ezetimibe at 10 mg/kg/day, cholesterol absorption was similar to that seen in the untreated knockout mice (16.2 ±0.6% compared to 15.6% ±0.4%, respectively). Thus, the majority of

cholesterol absorption is dependent on the presence of NPC1L1 and the residual cholesterol absorption in mice lacking NPC1L1 is insensitive to ezetimibe treatment. These results indicate that NPC1L1 is involved in the small intestinal enterocyte uptake and absorption of cholesterol and is in the ezetimibe sensitive pathway.

Example 25: Mouse Screening Assay (Acute Cholesterol Absorption).

The following screening assay is used to identify the presence of an NPC1L1 antagonist in a sample.

Mice deficient in *NPC1L1* (-/-) are generated by breeding heterozygote mice (+/) to obtain wild-type (+/+) and *NPC1L1* deficient mice (-/-).

In a first set of experiments, non-fasted mice (6.5-9 weeks old, mixed 129 and C57BL/6 background) are weighed and grouped (n=1 to 4 -/- and n=1 to 4 +/+). All animals are gavaged (Feeding needles, 24G x 1 inch, Popper and Sons, NY) with 0.1 ml corn oil (Sigma; St. Louis, MO) containing 1μCi ^{14}C-cholesterol (New England Nuclear, [$^{4\text{-}14}$C] Cholesterol, NEC-018) and 0.1mg carrier cholesterol mass (Sigma; St. Louis, MO).

In another set of experiments, 1 to 4 wild-type *NPC1L1* mice (+/+) are treated identically to the mice in the first set of experiments, above, except that the mice are additionally fed a sample to be tested for the presence of an NPC1L1 antagonist.

Two hours later, blood is collected from each mouse by heart puncture. The liver is removed, weighed, and three samples are placed into 20 ml counting vials. Tissues are digested in 1 ml of 1N NaOH at 60°C overnight. The tissue digests are acidified by addition of 250μl of 4N HCl prior to liquid scintillation counting (LSC). Plasma is isolated by centrifugation at 10,000 rpm for 5 minutes in a microfuge and duplicate 100μl aliquots of plasma are taken for LSC.

Cholesterol absorption, evaluated by this acute technique is expressed as the total amount of radioactive cholesterol in the plasma and liver. The sample tested is determined to contain an NPC1L1 antagonist when the level of cholesterol absorption (as measured by the above described methods) in the wild-type *NPC1L1* mouse (+/+) which was fed the sample and in the *NPC1L1* deficient mouse (-/-) are less than the amount of cholesterol absorption in the wild-type *NPC1L1* mouse (+/+) which was not fed the sample.

Example 26: Mouse Screening Assay (Fecal Ratio Method: Cholesterol/Sitostanol).

The following screening assay is used to identify the presence of an NPC1L1 antagonist in a sample.

Cholesterol absorption in the mice is determined by the dual fecal isotope ratio method as described by Altmann *et al.* (Biochim. Biophys. Acta. 1580(1): 77-93 (2002)).

Three groups of mice (n=1-6/group) are assembled. Two separate groups comprise wild-type *NPC1L1* mice (+/+) and one group comprises *NPC1L1* deficient mice (-/-).

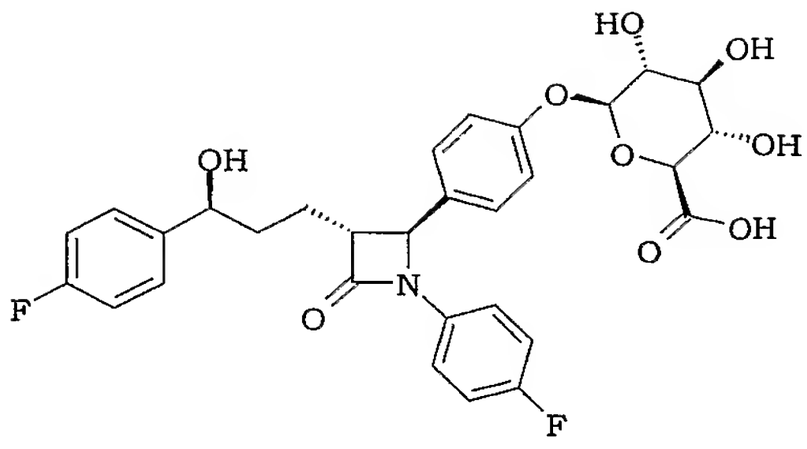
Each group is fed a standard rodent chow diet and in some groups treated daily. Mice are gavaged with ^{14}C-cholesterol (1μCi, 0.1mg unlabeled cholesterol) and ^{3}H-sitostanol (2μCi) in 0.1ml corn oil. One group of mice, which comprise wild-type NPC1L1 mice (+/+) are further fed a sample to be tested for the presence of an NPC1L1 antagonist. Feces are collected for 2 days and fecal ^{14}C-cholesterol and ^{3}H-sitostanol levels are determined by combustion in a Packard Oxidizer.

The sample tested is determined to contain an NPC1L1 antagonist when the level of cholesterol and/or sitostanol absorption (as measured by the above described methods) in the wild-type *NPC1L1* mouse (+/+) which was fed the sample and in the *NPC1L1* deficient mouse (-/-) are less than the amount of cholesterol and/or sitostanol absorption in the wild-type *NPC1L1* mouse (+/+) which was not fed the sample.

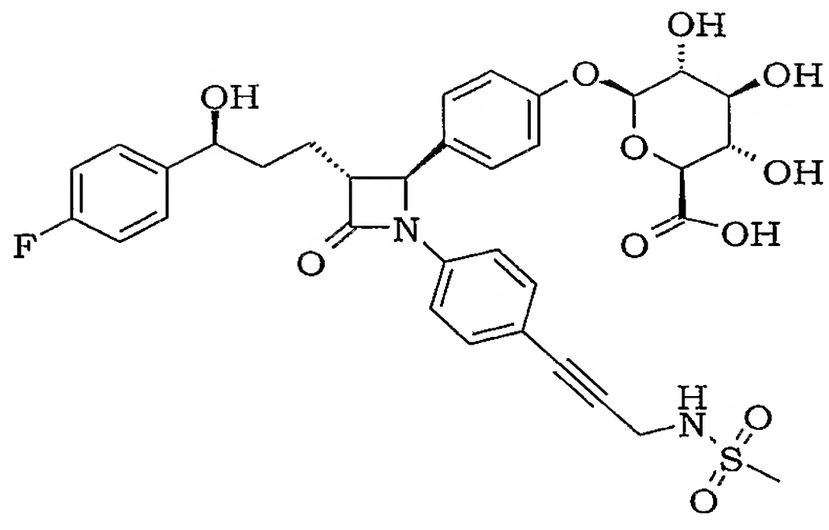
Example 27: Binding Analysis Using Brush Border Membrane Vesicles

The following screening assay may be used to identify the presence of an NPC1L1 ligand in a sample.

Materials. The following two compounds were synthesized for the binding assay described herein , ^{3}H-ezetimibe glucuronide **1** (34.5 Ci/mmol) and its ^{35}S-propargyl-sulfonamide derivative **2** (800-1100 Ci/mmol).

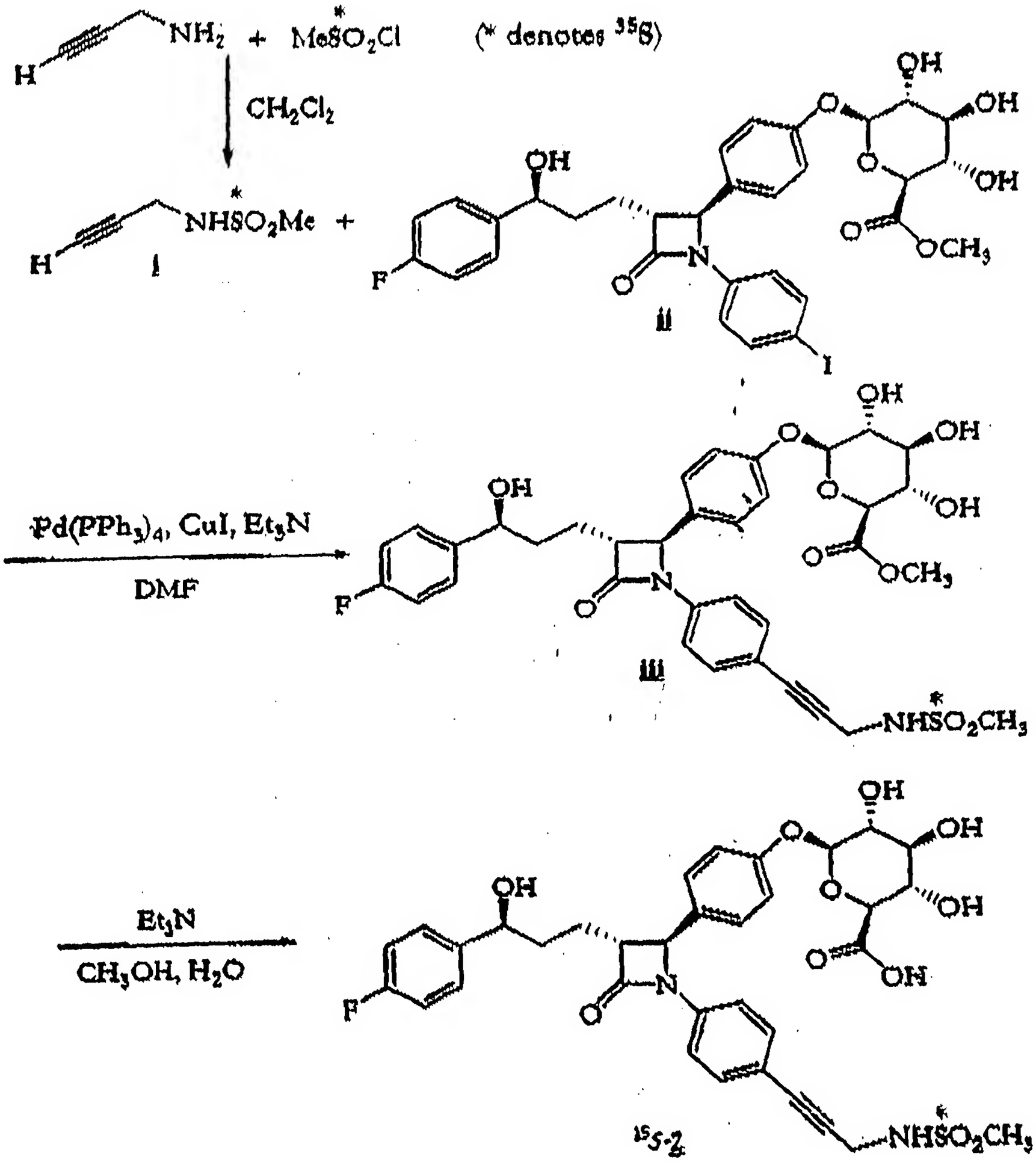


Ezetimibe-glucuronide
1



S-propargyl-sulfonamide
ezetimibe-glucuronide
2

Synthesis of ezetimibe glucuronide and S-propargyl-sulfonamide ezetimibe-glucuronide. Ezetimibe glucuronide (compound **1**) (also referred to as EZE-glucuronide) can be made according to the procedures in U.S. Patent No. 5,756,470. The general scheme below illustrates a method for the synthesis of compound **2** and radiolabel led ^{35}S-**2**.


NH2 + MeSO2Cl
(* denotes 35S)
CH2Cl2
NHSO2Me +
I
II
III
Pd(PPh3)4, CuI, Et3N
DMF
Et3N
CH3OH, H2O
NHSO2CH3

Preparation of compound ^{35}S-**2** (Compound **2** with radiolabelled ^{35}S)

Step A: Preparation of [^{35}S]N-prop–2-yn-1-ylmethanesulfonamide (**i**). The appropriate volume of [^{35}S]methane sulfonyl chloride (see Dean, D.C.; et al., *J. Med. Chem.* **1996**, *39*, 1767) totaling 3.5 mCi was removed from a stock solution in methylene chloride and placed in a 5mL conical flask. It was then distilled at atmospheric pressure until the volume was approximately 50 µL. To this solution was immediately added 50 µL of propargylamine. After 15 min, the reaction mixture was diluted with 10 mL of ethyl acetate, washed with saturated sodium bicarbonate solution (3 x 2 mL), and dried over sodium sulfate. After filtration the resulting solution had a count of 3.3 mCi and a radiochemical purity of 99.9 % by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 5 % acetonitrile:H_2O (0.1 % TFA) to 100 % acetonitrile, 15 min linear gradient, 1 mL/min, t_R = 4.4 min).

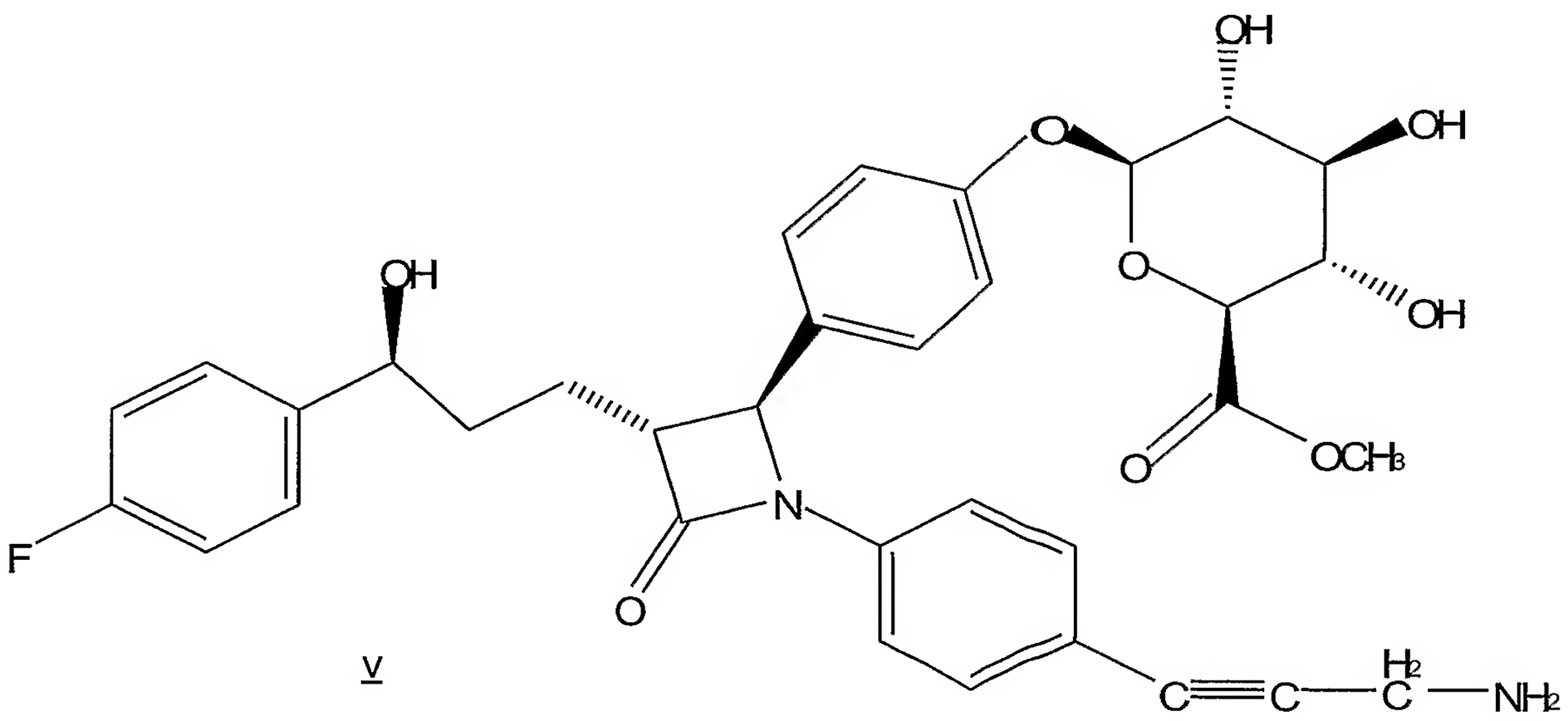
Step B: Preparation of [^{35}S] 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate ([^{35}S]) (**iii**). Dissolved 3.0 mCi of [^{35}S]*N*-prop-2-yn-1-ylmethanesulfonamide, 1 mg of compound **ii** (prepared according to Burnett, D.S. et al., Bioorg. Med. Chem. Lett. (2002), vol. 12, p. 311), and 1 µL of triethylamine in 100 µL of dimethylformamide inside a plastic microcentrifuge tube. To this solution was added 10 µL of a stock solution containing 8.1 mg of tetrakis(triphenylphosphine)palladium(0) and 1.4mg of copper iodide in 1 mL of dimethylformamide. Stirred at room temperature for sixty hours at which time HPLC indicated 55% conversion. This reaction mixture, which had a radiochemical purity of 44.4% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 5 % acetonitrile:H_2O (0.1 % TFA) to 100 % acetonitrile, 15 min linear gradient, 1 mL/min, t_R = 9.3 min) was taken on directly to the next step.

Step C: Preparation of [^{35}S] 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl β-D-glucopyranosiduronic acid ^{35}S-**2**. The crude reaction mixture containing compound **iii** was treated with 25 µL of methanol, 90 µL of water, and 30 µL of triethylamine and stirred at room temperature for one hour at which time it was concentrated to near dryness under a slow stream of nitrogen. The residue was dissolved in 1:1 acetonitrile:H_2O and subjected to semi-preparative chromatography

(Zorbax XDB C8 250 x 9.4 mm column, 70:30 acetonitrile:H_2O (0.1 % TFA) 4 mL/min, 1 x 0.2 mL injections). 540 μCi of product was obtained which had a radiochemical purity of 99.9% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 70:30 acetonitrile:H_2O (0.1 % TFA), 1 mL/min, t_R = 10.4 min) and coeluted with an authentic sample of compound **2**. LC/MS m/z = 508 (product – glucuronide – H_2O), SA = 769 Ci/mmol.

Alternate Preparation of ^{35}S-2.

Step A: Preparation of **iii.** The appropriate volume of [^{35}S]methane sulfonyl chloride (see Dean, D.C.; et al., *J. Med. Chem.* **1996**, *39*, 1767) totaling 1.3 mCi was removed from a stock solution in methylene chloride and placed in a 5mL conical flask. It was then distilled at atmospheric pressure until the volume was approximately 50 μL. To this solution was immediately added a solution of 1 mg of **v** in 5 μL of pyridine (freshly distilled over calcium hydride).



v

The solution was stirred at room temperature for five minutes at which time it was concentrated to near dryness under a slow stream of nitrogen. This reaction mixture, which had a radiochemical purity of 80.1% by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 5% acetonitrile:H_2O (0.1 % TFA) to 100 % acetonitrile, 15 min linear gradient, 1 mL/min, t_R = 9.3 min) was taken on directly to the next step.

Step B:Preparation of ^{35}S-**2.** The crude reaction mixture containing **iii** was treated with 25 μL of methanol, 90 μL of water, and 30 μL of triethylamine and

stirred at room temperature for one hour at which time it was concentrated to near dryness under a slow stream of nitrogen. The residue was dissolved in 1:1 acetonitrile:H_2O and subjected to semi-preparative chromatography (Zorbax XDB C8 250 x 9.4 mm column, 70:30 acetonitrile:H_2O (0.1 % TFA) 4 mL/min, 1 x 0.2 mL injections). 350 μCi of product was obtained which had a radiochemical purity of 98.4 % by HPLC (Zorbax XDB C8 column, 4.6 x 150 mm, 70:30 acetonitrile:H_2O (0.1 % TFA), 1 mL/min, t_R = 10.4 min) and coeluted with an authentic sample of **2**. LC/MS m/z = 508 (product – glucuronide – H_2O), SA = 911 Ci/mmol.

Following the same general procedure for synthesis of ^{35}S-**2**, except omitting the radiolabel ling, compounds **2** and **iv** can be prepared.

Preparation of brush border membrane vesicles (BBMV).

Membranes were prepared from Rhesus macaque (Macaca mulatta), rat (male Sprague-Dawley), and mouse (male C57BL/6J) intestines, using Mg^{++} precipitation method described in the following references and with modifications described below (Hauser, H., Howell, K., Dawson, R.M.C., Bowyer, D. E. Biochim. Biophys. Acta 602, 567-577 (1980); Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal, K., Muller, G., Tripier, D., Wess, G. J. Biol. Chem. 268, 18035-18046 (1993); Rigtrup, K.M., Ong, D.E. Biochemistry 31, 2920-2926 (1992)).

The intestines from freshly sacrificed animals were cut into segments, perfused with ice-cold saline buffer (Buffer A: 26 mM $NaHCO_3$, 0.96 mM NaH_2PO_4, 5 mM HEPES, 5.5 mM glucose, 117 mM NaCl, 5.4 mM KCl, pH = 7.4), placed on cold glass plates, opened longitudinally, and the mucosa scraped with glass microscope slips. This mucosa could be used fresh or frozen with identical results.

To prepare the membranes, the mucosal scrapings were resuspended in 20 volumes of cold buffer consisting of 300 mM D-mannitol, 5 mM EGTA, 12 mM Tris, pH 7.4 with HCl, and containing 0.1 mM PMSF and a 1% dilution of a protease inhibitor cocktail (set 1, Calbiochem). They were homogenized using a Polytron at medium speed on ice until inspection with a microscope indicated complete cell lysis. Then, solid $MgCl_2$ was added slowly with stirring to a final concentration of 10 mM, and the solution was kept stirring on ice for 15 min. Cellular debris was removed by centrifugation for 15 min at 3,000g, and the membranes were recovered by centrifugation for 60 min at 48,000g. The membranes were further rinsed by re-suspension in a buffer containing 50 mM D-mannitol, 5 mM EGTA, and 2 mM Tris

at pH 7.40, and centrifugation for 60 min at ~48,000g. The final pellet was resuspended in 120 mM NaCl and 20 mM Tris at pH 7.40 to a concentration of ~10-20 mg protein/ml, aliquoted, frozen in liquid nitrogen, and stored at –80C. The activity was stable indefinitely and could be freeze-thawed with minimal loss of activity.

Membrane protein was measured by the Bradford assay (Bradford, M.M. Anal. Biochem. 72, 248-254 (1976)) using bovine serum albumin as standard. The enrichment in brush border membranes was assessed using gamma-glutamyltransferase as a marker enzyme (Kramer, W., Girbig, F., Gutjahr, U., Kowalewski, S., Jouvenal, K., Muller, G., Tripier, D., Wess, G. J. Biol. Chem. 268, 18035-18046 (1993)), which indicated a 6-fold enrichment over the initial homogenate.

Binding assay. Assays were conducted in 12 x 75 mm glass test tubes and total volume 20-100 ul. In general, frozen membranes were diluted in buffer A or buffer A containing 0.03% taurocholate and 0.05% digitonin to a final concentration of 0.02 to 5 mg/ml. Radiolabeled ligands were typically 25-50 nM for ^{3}H-ezetimibe (EZE)glucuronide **1**, and 3-5 nM for its ^{35}S analog **2**, in the assay, and they were delivered as DMSO or CH_3CN solutions. Competing ligands were likewise added as DMSO solutions to give a total 2-10 % organic solvent content. Nonspecific binding was defined by competition with 100 uM ezetimibe glucuronide. At least 2 components of buffer A, the bicarbonate and phosphate salts, were later found to be inconsequential and were routinely omitted. To ensure equilibrium was established, the reactions with compound **1** were incubated at least 3 hours for rhesus membranes and at least one hour for rat membranes at room temperature, and with compound **2** as long as 2 hours at 37°C with rhesus and rat brush membranes. Additionally, reactions with compound **2** were incubated as long as 2 hours at 37°C with membranes from HEK-293 cells expressing mouse, rat or human NPC1L1.

Bound ligand was quantified by single-tube vacuum filtration using GF/C glass fiber filters. Glass fiber filters (GF/C) were obtained from Whatman. The filters were pretreated by soaking with 0.5% polyethylenimine to reduce nonspecific binding. Filtration was accomplished by adding 2.5 ml of ice cold buffer (120 mM NaCl, 0.1% sodium cholate, and 20 mM MES at pH 6.70) to the assay tube, pouring the mixture through the filter, and then rinsing the tube and filter twice more with

another 2 x 2.5 ml buffer. The filters were counted in 7 ml vials using Packard DM liquid scintillation fluid. Where triplicate assays were performed, the standard error was typically <4%. As an example, a 100µl assay of rat brush border membranes at 2 mg/ml in the presence of 400,000 dpm (50 nM) ^{3}H-ezetimibe glucuronide gave 15,000 dpm specific and 3,000 dpm nonspecific binding. The filters contributed most of the nonspecific binding (2,000 dpm).

Alternatively, vacuum filtration of compound **2** on a Millipore 96-well plate (Whatman GF/C) can also be used to achieve adequate precision.

Data analysis. Data from saturation experiments were subjected to a Scatchard analysis, and linear regression was performed to yield the equilibrium dissociation constant (K_d) and maximum receptor concentration (B_{max}). Correlation coefficients for these determinations were typically greater than 0.96. Data from competition experiments were analyzed and IC_{50} values determined from Hill plots of the binding data. The kinetic data for ligand association and dissociation were subjected to the analysis of Weiland and Molinoff (Weiland, G., Molinoff, .B. Life Sci. 29, 313-330 (1981)). The dissociation rate constant for (k_{-1}) was determined directly for a first order plot of ligand dissociation versus time. The rate of ligand association (k_1) was determined from the equation $k_1 = k_{obs}([LR_e]/([L]\ [LR]_{max}))$ where [L] is the concentration of ligand, $[LR_e]$ is the concentration of the complex at equilibrium, $[LR]_{max}$ is the maximum number of receptors present, and k_{obs} is the slope of the pseudo-first order plot $L_n\ ([LE]_e/([LR]_e - [LR]_t))$ versus time.

Binding analysis. Ezetimibe is rapidly converted to its glucuronide *in vivo*, and this metabolite is thought to be largely if not exclusively responsible for inhibition of cholesterol uptake. Accordingly, both ^{3}H-ezetimibe and its corresponding glucuronide derivative (**1**) were prepared and tested for binding to intestinal brush border membrane preparations, using a single-tube vacuum filtration technique. As a result of the hydrophobic nature of ^{3}H-ezetimibe, high nonspecific binding was observed, precluding its use as a radioligand for the binding assay. However, due to the improved physical properties of the glucuronide derivative (**1**), specific binding was observed with this radioligand and it was used to assess binding in rhesus, rat, and mouse intestinal brush border membranes.

Rhesus, rat, and mouse intestinal scrapings were homogenized and the brush border membranes isolated. Specific binding was observed exclusively in the

membrane fraction. Plots of total, nonspecific, and specific binding to rhesus (Figure 1) and rat (Figure 2) brush border membranes. Aliquots of rhesus BBMV (83 µg/assay) or rat BBMV (250µg/assay) were incubated with increasing concentrations of ^{3}H-EZE-glucuronide. Total binding and nonspecific binding determined in the presence of 10-100 uM EZE-glucuronide are shown. Specific binding was calculated from the difference between total and nonspecific binding. Data were fit by nonlinear regression as described above, and the linear Scatchard plot is shown. In rhesus membranes, the data correspond to a single binding site with K_d = 41 nM and a concentration of 5.5 pmol/mg membrane protein. The affinity is ~10-fold lower in rat membranes (K_d = 540 nM). ^{3}H-EZE-glucuronide is not the best ligand for a binding assay for the mouse target due to the compounds low affinity in mouse membrane. These potencies correlate roughly with the sensitivity of these species to ezetimibe inhibition of cholesterol uptake.

Rate constants for binding and dissociation. Ezetimibe-glucuronide is slow to bind, and forms a relatively long-lived complex with its receptor. Indeed, this was key to detecting the interaction in a traditional filter-binding assay, as ligand/receptor interactions with K_d values greater than 100 nM often go unrecognized because of the typical fast off-rates of the ligands. Rate constants for association (k_{on}) and dissociation (k_{off}) of compound **1** were determined for rat and rhesus membranes, and used as an alternative method to calculate the dissociation constant (K_d) according to the relationship $K_d = k_{off}/k_{on}$. 300µg/assay of rat brush border membrane vesicles were incubated with 25nM ^{3}H-EZE-glucuronide at room temperature for up to three hours for the association kinetic studies. 83 µg/assay of rhesus brush border membrane vesicles were incubated with 25nM ^{3}H-EZE-glucuronide at room temperature for up to five hours for the association kinetic studies. Nonspecific binding measured in the presence of 100µM EZE-glucuronide was subtracted from the total binding to calculate the specific binding shown in Figures 3A and 4A. For the dissociation kinetic study, rat brush border membrane vesicles were incubated with 25nM ^{3}H-EZE-glucuronide for 2 hours at room temperature and ligand dissociation was initiated by the addition of 100µM EZE-glucuronide. Rhesus brush border membrane vesicles were incubated with 42nM ^{3}H-EZE-glucuronide for 4 hours at room temperature and ligand dissociation was initiated by the addition of 100µM EZE-glucuronide. For both rat and rhesus

dissociation studies, samples were collected at various times and radiolabel was detected. Dissociation curves are shown in Figures 3B (rat) and 4B (rhesus).

For rat membranes, the rate constant for association is $\mathbf{k_{on} = 5{,}540\ M^{-1}\ s^{-1}}$ (compared to 10^8 to $10^9\ M^{-1}\ s^{-1}$ for diffusion controlled encounter), and the rate constant for dissociation is $k_{off} = 2.4 \times 10^{-3}\ s^{-1}$, corresponding to a half-life of 4.7 min. The data are shown in Figure 3, where the solid lines are theoretical for these rate constants. The K_d value predicted from these rate constants ($K_d = k_{off}/k_{on} = 440$ nM) agrees well with that measured at equilibrium ($K_d = 540$ nM).

For rhesus membranes, where ^{3}H-ezetimibe glucuronide is at least 10-fold more potent (as described above), the association rate remains the same but the half-life for dissociation of the complex increases to ~90 min. These data are shown in Figure 4, where the theoretical lines correspond to $\mathbf{k_{on} = 3{,}900\ M^{-1}\ s^{-1}}$ and $k_{off} = 1.23 \times 10^{-4}\ s^{-1}$, and predict $K_d = 32$ nM compared to that measured at equilibrium ($K_d = 41$ nM).

Example 28: Binding Analysis of a Potent NPC1L1 ligand

A ^{35}S-labeled propargyl-sulfonamide analogue of ezetimibe glucuronide (^{35}S-**2**) was identified as a potential NPC1L1 antagonist. Compound **2** was prepared as described in Example 27 and found to have markedly improved affinity for some species of brush border membranes vesicles. For rhesus brush border membranes vesicles, 56 μg protein/assay were incubated with 25 nM ^{3}H-EZE-glucuronide in the presence of increasing concentrations of EZE-glucuronide and **2**. For rat brush border membranes vesicles, 150 μg protein/assay were incubated with 50 nM ^{3}H-EZE-glucuronide in the presence of increasing concentrations of EZE-glucuronide and **2**. For mouse brush border membranes vesicles, 20 μg protein/assay were incubated with 3 nM ^{35}S-**2** in the presence of increasing concentrations of EZE-glucuronide and **2**.

2 is more potent against enterocyte brush border membrane preparations from rats (35-fold), but is equipotent with ezetimibe glucuronide for rhesus membrane preparations (Figure 5, Table 8). It also has enhanced affinity for mouse membranes (Figure 6, Table 8).

Table 8. Summary of inhibition constants (K_i) for binding of ezetimibe glucuronide 1 and its propargyl-sulfonamide derivative 2 to rhesus, rat, and mouse intestinal brush border membranes.

Compound	Rhesus	Rat	Mouse
1	39	530	2,300
2	38	15	144

K_i values are nM.

Example 29: Distribution of ^{3}H-ezetimibe glucuronide (1) binding to intestinal tissues.

Previous studies have established that cholesterol absorption occurs primarily in the jejunum, and is substantially lower in the ileum and duodenum. To determine if the binding activity is similarly distributed, the binding assay using ^{3}H-ezetimibe glucuronide (^{3}H-**1**) as a radioligand was used to determine the distribution of binding sites in sections from rhesus and rat intestines.

For the rhesus studies, 10 cm corresponding to the ileum of a rhesus small intestine was separated and the remaining intestine was divided into three segments, (proximal, middle and distal) of equal length (70 cm each). For the rat studies, 10 cm corresponding to the ileum of a rhesus small intestine was separated and the remaining intestine was divided into three segments, (proximal, middle and distal) of equal length (36 cm each). Brush border membrane vesicles were prepared as described in Example 27. Aliquots of vesicles (100-200μg) protein/assay were incubated with 50nM ^{3}H-EZE-glucuronide in the absence and presence of 100μM EZE-glucuronide.

As shown in Figure 7, specific binding for ^{3}H-ezetimibe glucuronide peaks in the jejunum in both species, consistent with the previously observed pattern of cholesterol absorption.

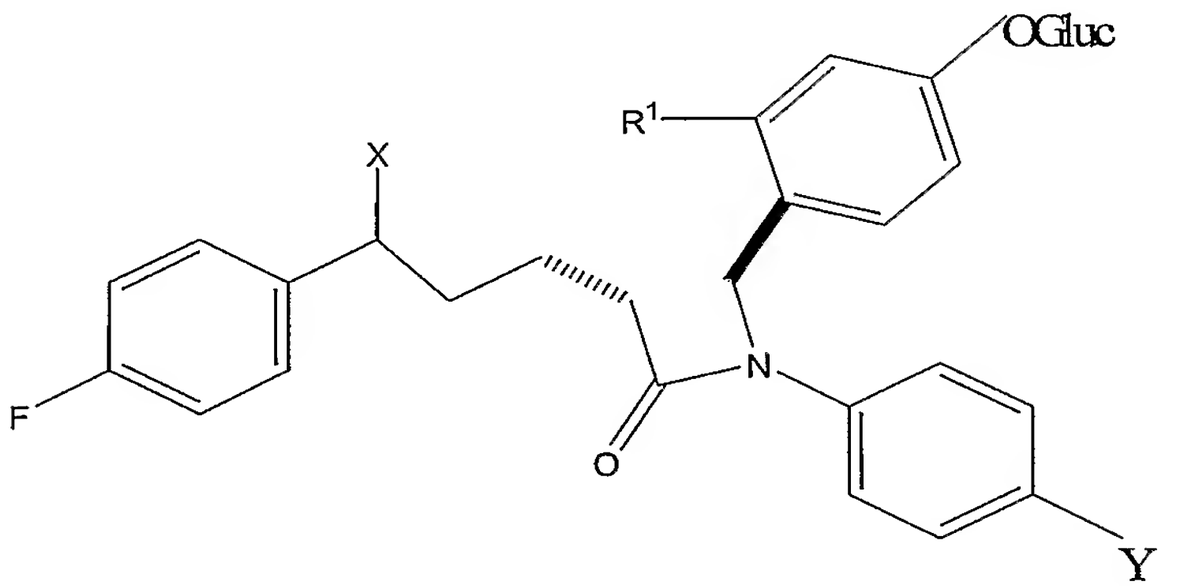
Example 30: Correlation of in vitro and in vivo binding activity of NPC1L1

To determine if *in vitro* binding activity is predictive of *in vivo* efficacy, the enantiomer of ezetimibe glucuronide and several close structural analogues of ezetimibe glucuronide that were tested in the rat membrane binding assay were tested in an acute rat cholesterol absorption study as described in Examples 23-26. The selected analogs had a range of *in vitro* potencies, and were

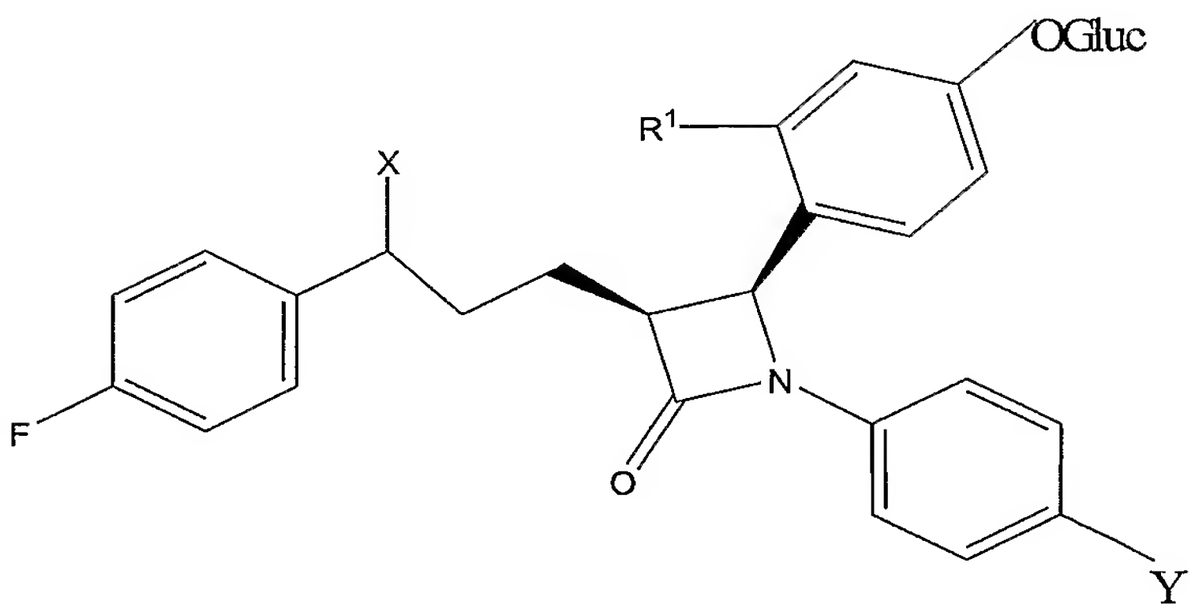
anticipated to have similar physical properties to ezetimibe glucuronide (Tables 9 and 12). The enantiomer, which has a Kd > 100,000 nM for the rat target, was inactive in the *in vivo* assay. For the other analogs, the same rank order of potency is observed in the *in vitro* and *in vivo* assays, further evidence that the observed binding is due to the target of ezetimibe.

Table 9. IC_{50} values of EZE-gluc and analogs to inhibit binding of 3H-EZE-gluc to rat brush border membrane vesicles.

Compound Name	R^1	X	Y	RAT IC_{50} (nM)
3	H	H2	F	2,300
EZE-gluc **1**	H	(S)-OH	F	530
EZE-gluc enantiomer **4**	H	(R)-OH	F	>100000
5	H	(R)-OH	F	3,900
6	H	=O	F	70,000
7	OH	(S)-OH	F	252



compounds **1**, **3**, **5**, **6** and **7**.



Backbone structure for compound **4**.

Example 31: Binding affinities of ezetimibe glucuronide and its analogs to recombinant NPC1L1

NPC1L1 was identified as a candidate target of ezetimibe from a search of genetic databases for cholesterol binding motifs. Subsequently, *NPC1L1* deficient mice were found to have 80% reduction of cholesterol absorption, and did not respond to ezetimibe treatment, strongly suggesting that this protein is required for ezetimibe efficacy. To determine if NPC1L1 is the direct target of ezetimibe, binding affinities were compared for ezetimibe glucuronide and several analogs to NPC1L1 transfected cells and rat brush border membrane vesicles.

Rat NPC1L1 transfected CHO cells (~500,000 cells/assay) were incubated with 5 nM ^{35}S-**2** (~1 million dpm/assay) for 2 hours at 37 °C in the absence or presence of increasing concentrations of EZE-glucuronide (compound **1**), compounds **2**, **3**, **5**, **6**, or **8**. Compound **8** is an analog of compound **2** wherein the hydroxyl group in the 3-hydroxylpropyl moiety of **2** is replaced with an oxo group.

Human NPC1L1 transfected CHO cells (~600,000 cells/assay) were incubated with 5 nM ^{35}S-**2** (~1 million dpm/assay) in buffer A for 2 hours at 37 °C in the absence or presence of increasing concentrations of EZE-glucuronide (compound **1**), compounds **2**, **3**, **5**, **6**, or **8**.

As shown in Figures 9 and 12, and Table 10, the affinities for the recombinant and native proteins are virtually identical, providing compelling evidence that NPC1L1 is the direct target of ezetimibe in mammalian tissues, and that other proteins are not required for binding.

Affinities of ezetimibe glucuronide and analogues thereof were also determined for human recombinant NPC1L1. The results, shown in Figure 9, indicate that ezetimibe glucuronide (**1**) has an affinity for the human protein of 907 nM. The propargyl-sulfonamide analogue (**2**) is approximately 50-fold more potent, with a K_d = 21 nM, suggesting that this compound has the potential for enhanced potency of cholesterol absorption inhibition in man.

Table 10: Comparison of inhibition constants (Ki) for binding to native rat intestinal brush border membranes and membranes from rat NPC1L1 transfected cells.

Analog	Recombinant rat NPC1L1 Ki, nM	Rat BBMV Ki, nM
EZE-glucuronide **1**	790	600
2	12	15
3	2400	2300
6	84500	70000
5	5800	3900
8	556	818

Example 32: Binding of ^{35}S-2 to membranes from wild type and NPC1L1 deficient mice.

Final confirmation that NPC1L1 is the target of ezetimibe was provided by binding studies with ^{35}S-**2** in intestinal brush border membranes from NPC1L1 deficient and control mice.

Brush border membranes vesicles were prepared from intestinal tissues of wild type and *NPC1L1* knockout (-/-) mice. 15, 30 and 60μg protein/ assay of brush border membranes vesicles were incubated with 4nM ^{35}S-**2** in buffer A for 3 hours at 37°C in the presence and absence of 100μM EZE-glucuronide.

The results, shown in Figure 10, indicate that no detectable binding is observed in membranes from NPC1L1 deficient mice, whereas age matched wild type control membranes have detectable binding. The binding affinity observed in this experiment in control mouse membranes (K_d=156 nM) was virtually identical to that observed in previous studies (Figure 11).

Example 33: Binding Analysis Using Brush Border Membrane Vesicles from Rat Mouse and Rhesus Monkey

Binding studies were performed to compare the relative binding affinity of ezetimibe glucuronide to various brush border membrane vesicles.

^{3}H-ezetimibe glucuronide **1** was prepared as described in Example 27. The brush border membranes were prepared as described in Example 27.

Binding Assay. Assays were conducted in 12 x 75 mm glass test tubes and total volume 20-100 µl. In general, frozen membranes were diluted in buffer A or buffer A containing 0.03% taurocholate and 0.05% digitonin to a final concentration of 0.5 to 5 mg/ml (Buffer A: 26 mM $NaHCO_3$, 0.96 mM NaH_2PO_4, 5 mM HEPES, 5.5 mM glucose, 117 mM NaCl, 5.4 mM KCl, pH = 7.4). Final concentrations of [^{3}H]ezetimibe glucuronide **1** were typically 25-50 nM , and were delivered as DMSO or CH_3CN solutions. Competing ligands were likewise added as DMSO solutions to give a total 1-5 % organic solvent content. Nonspecific binding was defined by competition with 100-500 µM ezetimibe glucuronide. At least three components of buffer A, the bicarbonate and phosphate salts, and glucose, were later found to be inconsequential and were routinely omitted. Reactions were incubated until equilibrium was achieved (one hour for rat or three hours for rhesus membranes).

Bound ligand was recovered by single-tube vacuum filtration on Whatman GF/C glass fiber filters. The filters were pretreated by soaking with 0.5% polyethylenimine to reduce nonspecific binding. Filtration was accomplished by adding 2.5 ml of ice cold buffer (120 mM NaC1, 0.1 % sodium cholate, and 20 mM MES at pH 6.7) to the assay tube, pouring the mixture through the filter, and then rinsing the tube and filter twice more with another 2 x 2.5 ml buffer. The filters were counted in 7 ml vials using Ultima Gold MV liquid scintillation fluid from Packard. Where triplicate assays were performed, the standard error was typically <4%. As an example, a 100µl assay of rat brush border membranes at 2 mg/ml in the presence of 400,000 dpm (50 nM) [^{3}H]ezetimibe glucuronide gave 15,000 dpm specific and 3,000 dpm nonspecific binding. The filters contributed most of the nonspecific binding (2,000 dpm).

Data Analysis. After correction for nonspecific binding, saturation-binding data were fit by nonlinear regression (Sigma Plot) to the single-site expression $[B] = B_{max} \times [L]/([L] + K_D)$. Linear Scatchard plots are shown for illustration. Data on K_i from competition experiments were analyzed by nonlinear regression to the expression $[B] = [B_O]/(1 + [I]/K_i^{obs})$, and where required were corrected for radioligand competition as $K_i = K_i^{obs}/(1 + [L^*]/K_D)$.

First-order rate constants (k_{obs} and k_{off}) were determined by nonlinear regression to the first order rate equation $A = A_o e^{-kt}$. Kinetic data for k_{on} were analyzed according to Weiland and Molinoff (32), using the equation $k_{on} = k_{obs}$ $([LR]_e/([L]\ [LR]_{max}))$, where [L] is the concentration of ligand, $[LR]_e$ is the concentration of the complex at equilibrium, $[LR]_{max}$ is the maximum number of receptors present, and k_{obs} is the apparent first-order rate constant.

Binding analysis. Binding studies using the [^{3}H]ezetimibe glucuronide a traditional rapid-filtration assay on glass fiber filters using enterocyte brush border membrane preparations from rat, mouse and rhesus monkey were performed (Table 11). Table 11 shows the binding affinities of [^{3}H]ezetimibe glucuronide to the membranes in the absence of detergents. The observed binding was specific, saturable, and consistent with a single molecular site. Scatchard analyses and the specific/nonspecific binding windows for rat and monkey are shown in Figure 12. The binding affinity is relatively weak in rat membranes (K_D = 542 nM) and even weaker in murine membranes (K_D = 10,000 nM). In contrast, binding affinity in rhesus monkey membranes is approximately 10-fold greater (K_D = 41 nM). The number of binding sites varied from 5 – 20 pmol/mg membrane protein depending on species and preparation.

The rates for binding and dissociation of [^{3}H]ezetimibe glucuronide were determined and found to be slow relative to those typically observed for protein-ligand interactions. For example, the rate constants for association to rat and monkey brush border membranes are k_{on} = 5.54 and 3.90 x 10^3 M^{-1} s^{-1} (Figure 12). These are 100,000-fold smaller than those typically observed for a diffusion controlled encounter, 10^8 to 10^9 M^{-1} s^{-1}. Similarly, these complexes are unusually long-lived, dissociating with rate constants of k_{off} = 2.4 x 10^{-3} s^{-1} and 1.2 x 10^{-4} s^{-1} at 25C, equivalent to half-lives of 4.7 and 96 min for the rat and monkey complexes, respectively. In comparison, half lives are normally <1 sec for dissociation of common diffusion controlled, 100 nanopolar K_D ligands. These rate constants predict K_D values ($K_D = k_{off}/k_{on}$) of 440 and 32 nM, respectively, which agree well with those measured by equilibrium titration (Figure 12), and by saturation as described earlier. Such slow-forming, long-lived complexes suggest that conformational changes in the protein are rate limiting.

Table 11: Comparison of ezetimibe binding affinity and cross species efficacy

Species	K_D (nM)	ED_{50} (mg/Kg)
mouse	12,000	0.5000
rat	542	0.0300
monkey	41	0.0005

Table 11 also shows a correlation between *in vitro* and *in vivo* binding of [^{3}H]ezetimibe glucuronide in various enterocyte brush border membrane preparations from rat, mouse and rhesus monkey. The *in vivo* ED_{50} values are derived from cholesterol absorption and cholesterol feeding studies. The rank order of ezetimibe potency (ED_{50}) *in vivo* as follows: rhesus (0.0005mpk) > rat (0.03mpk) > mouse (0.5mpk) is the same as the order of *in vitro* binding affinity (IC_{50}) as follows: rhesus monkey (41nM) < rat (542nM) < mouse (12,000nM).

The binding affinities of **1** to brush border membranes correlate well across species with the sensitivity to ezetimibe inhibition of cholesterol uptake *in vivo* (mouse < rat < monkey) (Clader, J. W. The discovery of ezetimibe; A view from outside the receptor. *J Med. Chem.* 47, 1-9 (2004); Davis, H.R. Jr., Compton, D.S., Hoos, L. & Tetzloff, G. Ezetimibe, a potent cholesterol absorption inhibitor, inhibits the development of atherosclerosis in ApoE knockout mice. *Arterioscler. Thromb. Vasc. Biol.* 21, 2032-2038 (2001); Burnett, D.A. Beta-lactam cholesterol absorption inhibitors. *Curr. Med. Chem.* 11, 1873-1887 (2004), consistent with the hypothesis that the assay is relevant to the target of ezetimibe *in vivo* (Table 11). As evidence that this interaction is very specific, the glucuronide of the enantiomer of ezetimibe was prepared and found to be completely inactive *in vitro* ($K_i > 100 \times K_D$ for ezetimibe glucuronide in all species), consistent with its lack of activity *in vivo* in a rat acute cholesterol absorption model (see Table 12 in which the enantiomer is analyzed).

Example 34: NPC1L1 as the target of ezetimibe in NPC1L1-expressing HEK293 cells.

This example demonstrates that ezetimibe binds specifically to NPC1L1-expressing HEK293 cells.

Transient expression of NPC1L1. Plasmid pCR3.1 expressing rat NPC1L1 (Genbank AY437867) or human NPC1L1 (Genbank AY437865) were

prepared using standard molecular biology protocols. HEK-293 cells (ATCC) were seeded at 10 x 10^6 cells per T-225 flask (Corning) in DMEM containing 10% fetal calf serum, 4.5 g/L D-glucose and L-glutamine, 18 hours prior to transfection. They were transiently transfected with 25 μg of DNA using Fugene transfection reagent (Roche Biochemical) at a ratio of 6:1 Fugene:DNA. Following transfection, the cells were incubated at 37°C and 5% CO_2 for 48 hours, and then harvested using PBS based cell dissociation buffer (Gibco), pelleted at 500 x g, snap frozen on dry ice, and stored at -80°C.

Membrane preparation from HEK-293 cells. Membranes were prepared by resuspending the frozen cell pellets in ten volumes of 20 mM HEPES/Tris buffer at pH 7.40 containing 8% sucrose, and sonicating the suspensions with a probe sonicator on ice until most of the cells were lysed. To isolate the membranes, the sonicates were centrifuged at 1600 x g for 10 min to remove cell debris, and then the supernatants were centrifuged at 125,000 x g for 1 hour to recover the membranes. These membranes were resuspended in 20 mM HEPES/Tris buffer at pH 7.40 containing 160 mM NaCl and 5% glycerol, and stored at 10-20 mg/ml protein at -80°C.

Pursuing the recent evidence indicating that NPC1L1 is an important component of the pathway inhibited by ezetimibe, recombinant rat and human NPC1L1 were expressed in human embryonic kidney (HEK) 293 cells (Figure 13, Panel 1). Cell lysates from HEK-293 cells expressing NPC1L1 (Lanes 1 and 3 of Panel 1 Figure 13) and wild-type cells (Lanes 2 and 4 of Panel 1 Figure 13) were analyzed by gel electrophoresis and Western blot with an anti-NPClL1 antibody A1801. An excess of NPClL1-specific peptide was included to assess specificity of the antibody for NPC1L1 (Lane 3 and 4 of Panel 1 Figure 13). Preliminary binding studies using **1** revealed specific binding to membrane preparations from cells expressing NPC1L1, and no specific binding to membranes from mock transfected cells (not shown).

Binding to NPC1L1 expressing cells was also observed with a BODIPY-labeled fluorescent ezetimibe glucuronide analog (SCH354909) (Figure 13, Panel 2A). Panel 2 of Figure 13 shows confocal microscope images of a fluorescent ezetimibe glucuronide analog (SCH354909) bound to the surface of NPC1L1-293 cells (Panel 2A), nonspecific binding of SCH354909 to NPC1L1-293 cells in the

presence of 100 μM unlabeled ezetimibe glucuronide (Panel 2B), binding of SCH354909 to wild type HEK 293 cells (Panel 2C), and nonspecific binding of SCH354909 to wild type HEK 293 cells in the presence of 100 μM unlabeled ezetimibe glucuronide (Panel 2D). In each case, plated cells were incubated in culture media with 500 nM SCH354909 for 4 hours at 37°C. Cells were subsequently washed with PBS and fluorescence was detected using confocal microscopy.

Binding of SCH345909 was clearly evident at the cell surface membrane of the NPC1L1-expressing cells and was completely abolished in the presence of excess unlabeled ezetimibe glucuronide (Figure 13, Panel 2C). No binding was observed in wild type HEK 293 cells (Figure 13, Panels 2B and 2D). These results demonstrated that ezetimibe glucuronide binds specifically to NPC1L1.

Example 35: NPC1L1 as the in vivo target of ezetimibe.

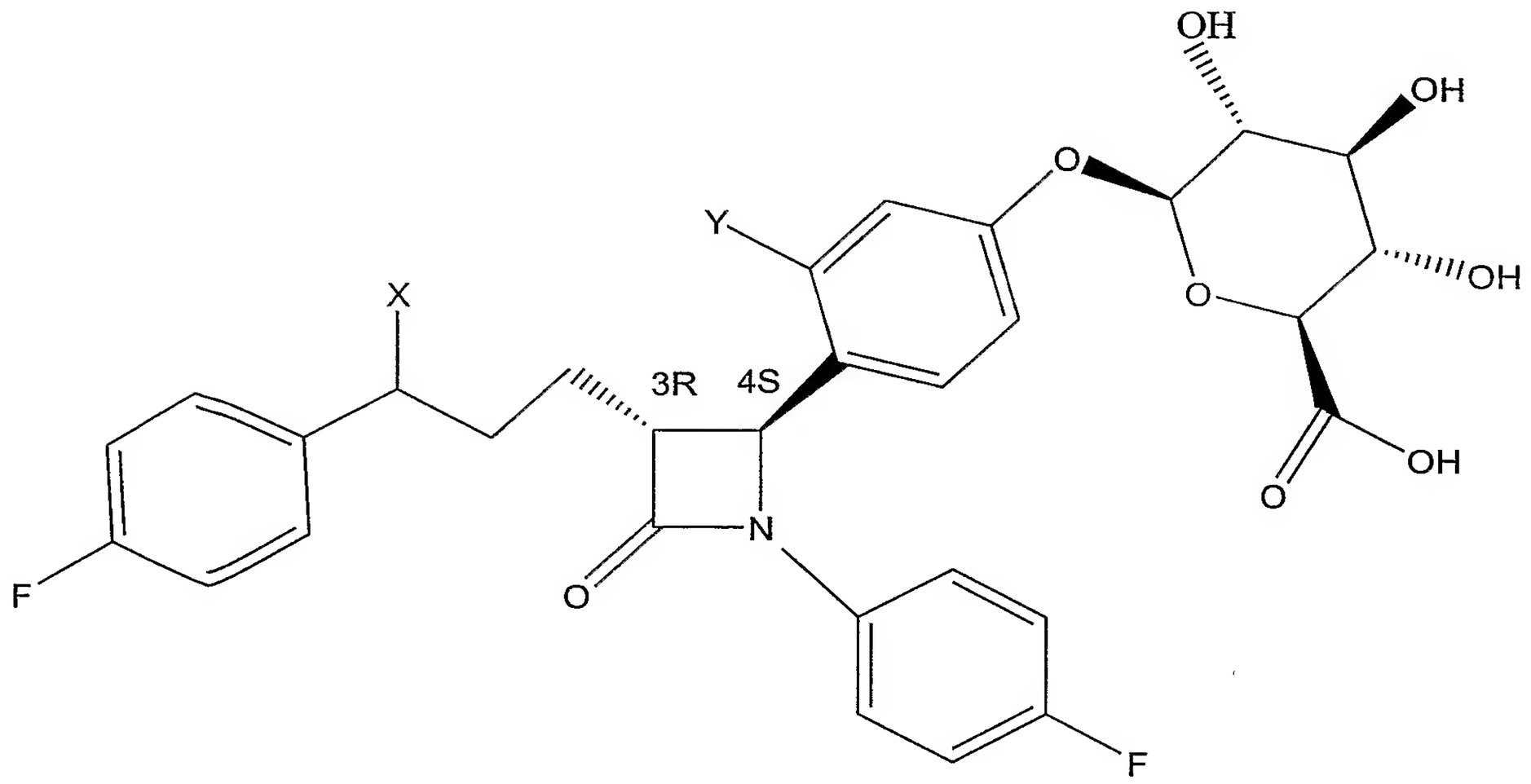
To obtain evidence that NPC1L1 is the direct binding target of ezetimibe *in vivo*, binding affinities of **1** and several key analogs were determined for recombinant rat and human NPC1L1 expressed in HEK-293 cell membranes and compared to those for native rat and rhesus intestinal enterocyte brush border membranes. A series of ezetimibe analogs was selected with subtle structural diversity, but with no binding affinities to native brush border membranes that covered a range of 1000-fold.

Table 12 shows a comparison of binding affinities (K_i values) for recombinant NPC1L1-293 cell membranes and native brush border membranes Selected analogs of ezetimibe glucuronide are compared against recombinant rat and human NPC1L1 membranes prepared from transiently transfected HEK-293 cells compared to native rat and rhesus brush border membranes. The binding assays were conducted in a final volume of 20 μl in the presence of 0.03% sodium taurocholate and 0.05% digitonin until equilibrium was achieved. 1.25 mg protein/ml and 100 nM **1** were used for native rat, recombinant rat, and recombinant human experiments, and 1.25 mg protein/ml and 20 nM **1** were used for native rhesus monkey experiments. Observed total and nonspecific binding, respectively, in the absence of inhibition were native rat: 7,700 & 1,100, recombinant rat: 33,000 & 1,100, native rhesus monkey: 7,300 & 367, and recombinant human: 19,200 & 1,000 dpm. Analog structures are defined in Table 12. Compound **4** has the stereochemical configuration 3S, 4R, and is the glucuronide of the enantiomer of ezetimibe. These determinations

were conducted in buffer containing 0.03% taurocholate and 0.05% digitonin, levels below the critical micelle concentrations of these detergents. These conditions enhanced apparent binding by as much as 20-fold for the recombinant preparations (principally a B_{max} effect), and greatly facilitated a quantitative comparison of K_i values for **1** and its analogs.

As shown in Table 12, the K_i values for recombinant rat NPC1L1 and native rat brush border membranes are virtually identical, strongly suggesting that NPC1L1 is the molecular target of ezetimibe *in vivo*. In the case of membranes from cells expressing recombinant human NPC1L1, the binding affinities also parallel those observed in rat membranes, whereas binding affinities for native rhesus brush border membranes are uniformly ~10-fold more potent. This result is consistent with the finding that ezetimibe is an order of magnitude more potent in monkey than in human or rat (Clader, J. W. The discovery of ezetimibe; A view from outside the receptor. *J Med. Chem.* 47, 1-9 (2004); Jeu, L. & Cheng, J.W. Pharmacology and therapeutics of ezetimibe (SCH 58235), a cholesterol-absorption inhibitor. *Clin. Ther.* 25, 2352-2387 (2003)).

TABLE 12



5

Analog	X	Y	Rat BBM Ki (nM)	Rat NPC1L1 Ki (nM)	Human NPC1L1 Ki (nM)	Monkey BBM Ki (nM)
EZE-gluc **1**	OH (*S*)	H	390	210	220	15
4*	OH (*R*)	H	130,000	74,000	130,000	22,000
3	H	H	1,600	820	1,000	150
6	=O	H	33,000	23,000	14,000	3,300
5	OH (*R*)	H	2,800	1,700	1,300	120
7	OH (*S*)	OH	280	360	210	60

* glucuronide of the enantiomer of ezetimibe has stereochemical configuration 3*S*, 4*R*

Conclusive evidence that NPC1L1 is the target of ezetimibe was provided by studies with tissues from NPC1L1 deficient mice. Enterocyte brush border membranes prepared from NPC1L1 deficient mice showed no detectable specific binding affinity for **1**, whereas membranes from age-matched wild-type mice showed a high level of specific binding with a $K_D = 12$ μM (Figure 14).

For Figure 14A, enterocyte brush border membranes were prepared from NPC1L1 deficient male mice and same sex wild-type littermates, and tested for binding of **1**. Conditions for binding were 5 mg/ml protein and 500 nM 1 in a volume of 20 μl and in the presence of 0.03% sodium taurocholate and 0.05% digitonin. Membranes from wild type mice are on the left and from NPC1L1 deficient mice on the right. The bar graphs indicate total binding (left bar), nonspecific binding in the presence of 500 μM cold ezetimibe glucuronide (middle bar), and specific (right bar) binding, respectively for each of wild type and NPC1L1 deficient mice, and error bars represent triplicate measurements. The graphs show that although specific binding is readily detectable in wild-type mice, it is absent in NPC1Ll-deficient mice.

Figure 14B shows a plot demonstrating competition of unlabeled ezetimibe glucuronide against 1. Membranes from wild-type mice (upper curve) gave $K_i = 12{,}000$ nM, while specific binding was virtually undetectable in membranes from the knockout animals (lower curve). Conditions were those described in Figure 14A.

The present studies involve a quantitative comparison of binding between recombinant proteins and brush border membranes. SR-BI (scavenger receptor type B1) was previously identified as a potential target using an expression cloning strategy employing ezetimibe binding to candidate proteins; this hypothesis was readily dismissed when neither cholesterol absorption nor ezetimibe activity were affected in SR-BI deficient mice (Altmann, S. W. *et al.* The identification of intestinal scavenger receptor class B, type 1 (SR-B1) by expression cloning and its role in cholesterol absorption. *Biochem. Biophs. Acta* 1580, 77-93 (2002)). The results show that ezetimibe binds to native intestinal membranes and cells expressing recombinant NPC1L1 with comparable affinity, and does not bind to membranes from NPC1L1 deficient mice, indicating a specific binding interaction between NPC1L1 and ezetimibe. Together with the previously published findings that mice deficient in NPC1L1 are defective in intestinal cholesterol uptake, and are no longer responsive to ezetimibe (Altmann, S. W. *et al.* Niemann-Pick Cl Like 1 protein is critical for

intestinal cholesterol absorption. *Science* 303, 1201-1204 (2004)), these data definitively establish NPC1L1 as the direct target of ezetimibe.

Example 36: Effect of detergents on [^{3}H]ezetimibe glucuronide binding.

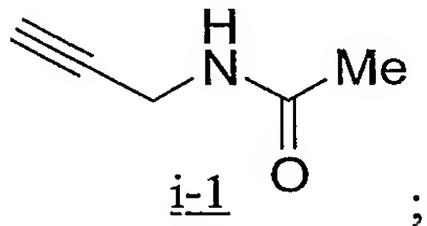
A practical aspect of work with the recombinant protein was that the number of binding sites in transfected NPC1L1-293 cell membranes initially appeared quite low. The influence of a combination of 0.03% taurocholate and 0.05% digitonin on specific binding to these and native enterocyte brush border membrane preparations is dramatic as illustrated in Figure 15.

Equal amounts (25 μg protein) of rat brush border membranes, membranes from HEK-293 cells transiently expressing recombinant rat and human NPC1L1, were incubated with 25 nM **1** in a final volume of 20 μl until equilibrium was achieved. The incubation conditions were buffer A with and without sodium taurocholate and digitonin to a final concentration of 0.03% and 0.05%, respectively. On the x-axis, "C" denotes controls in the absence of detergent, and "+det" the response in the presence of both detergents. The results are shown in 3 bar groupings; Total binding (left bar in each 3 bar group), nonspecific binding in the presence of 100 μM unlabeled ezetimibe glucuronide (middle bar in each 3 bar group), and specific binding (right bar in each 3 bar group) are shown.

Example 37: Binding affinities of ezetimibe glucuronide and various analogues NPC1L1 in rat and rhesus monkey membranes.

As determined from binding assay results using ^{3}H-ezetimibe glucuronide with rat brush border membrane, representative tested compounds of Formula II were determined to have IC_{50}'s of about 13,000nM or lower, and particularly certain tested compounds had IC_{50}'s of about 1900nM or lower, more particularly certain tested compounds had IC_{50}'s of about 1000nM or lower, and most particularly certain tested compounds had IC_{50}'s of less than 100nM. As determined from binding assay results using ^{3}H-ezetimibe glucuronide with rhesus brush border membrane, representative tested compounds of Formula II were determined to have IC_{50}'s of about 4200nM or lower, and particularly certain tested compounds had IC_{50}'s of about 165nM or lower, more particularly certain tested compounds had IC_{50}'s of less than 100nM, and most particularly certain tested compounds had IC_{50}'s of less than 50nM.

The designations below are used in the Examples that follow for certain repetitively used intermediates: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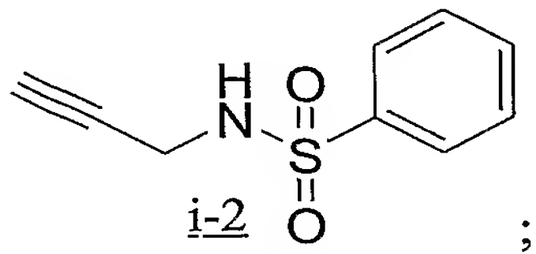
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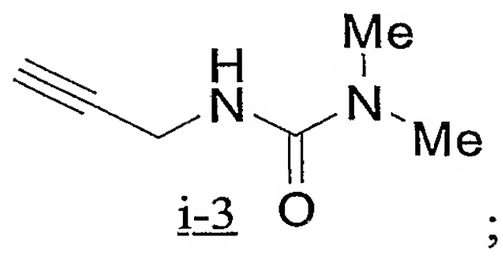
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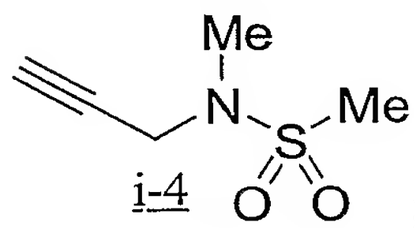
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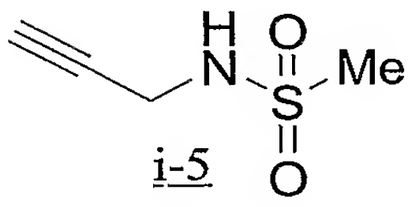
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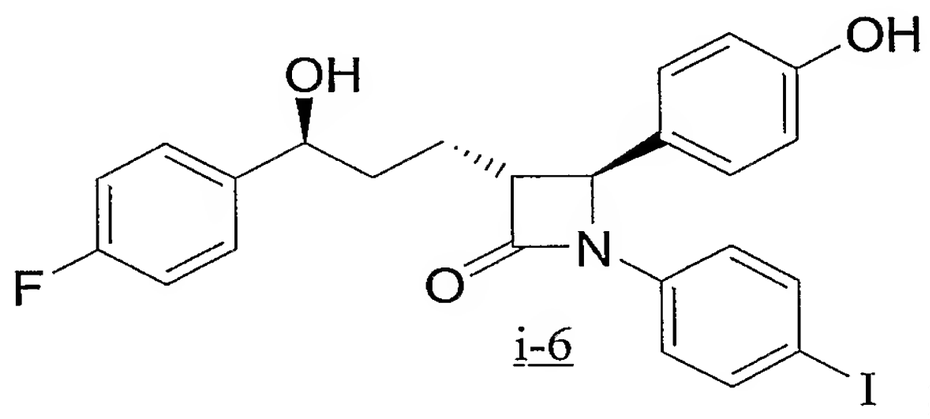
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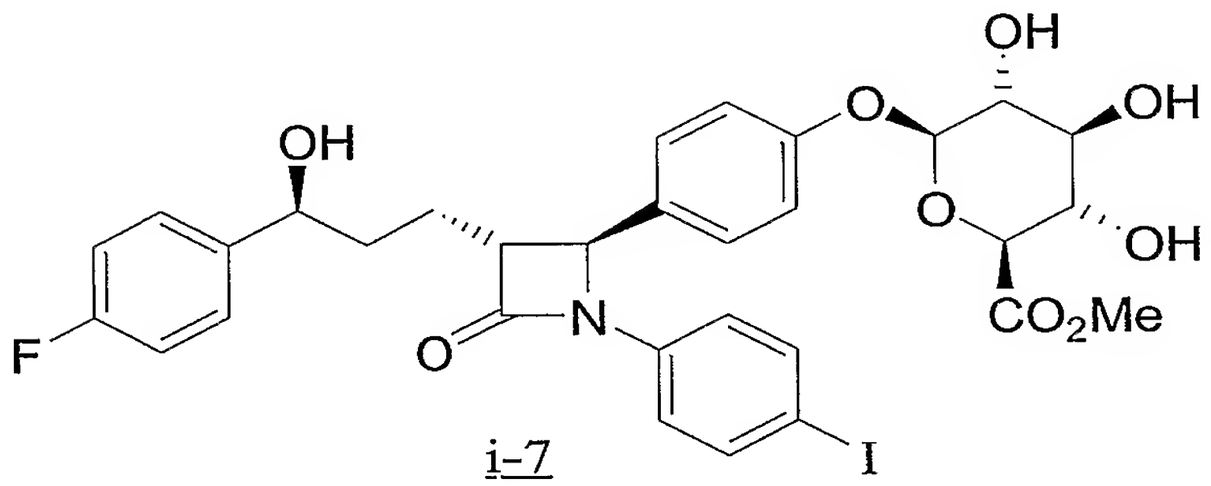
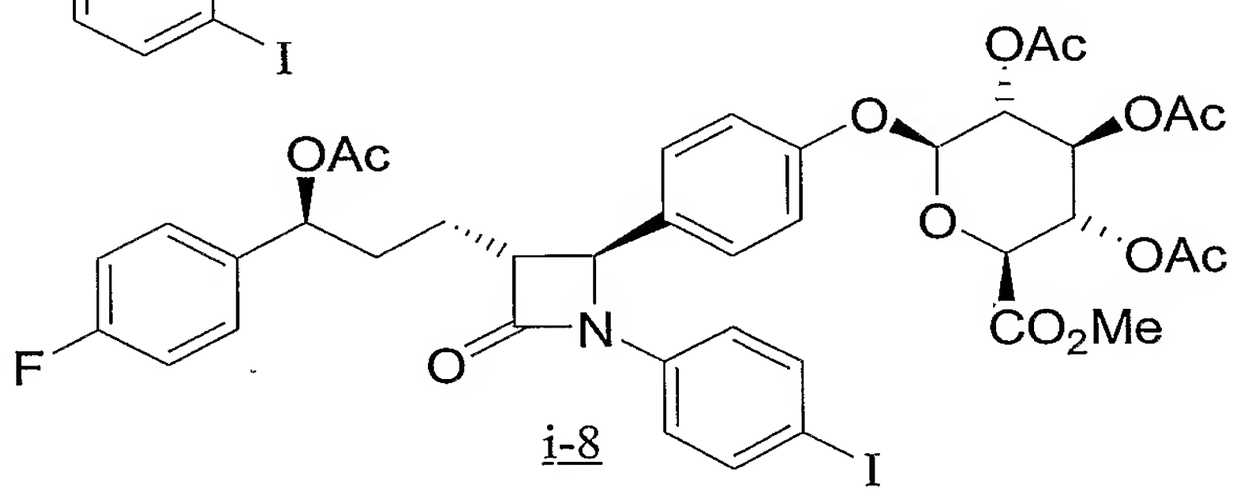
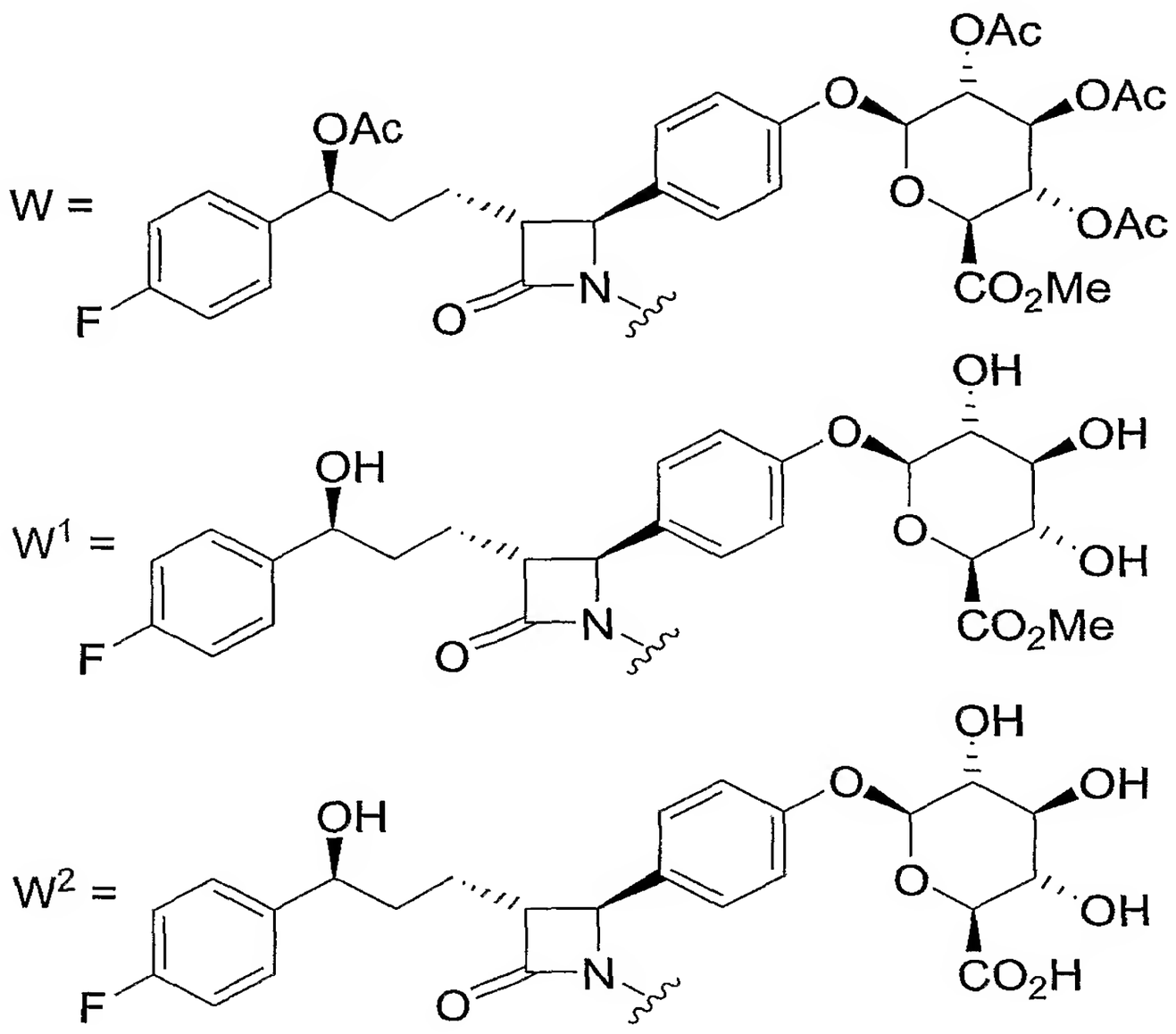

i-7


i-8

The compounds (3*R*,4*S*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-1-(4-iodophenyl)azetidin-2-one (i-6) and 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-iodophenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate (i-7) were prepared according to Burnett, D. S.; Caplen, M. A.; Domalski, M. S.; Browne, M. E.; Davis, H. R. Jr.; Clader, J. W. *Bioorg. Med. Chem. Lett.* (2002), *12*, 311. Compound i-8 is the hydroxy-protected analog of i-7, where the protecting group is acyl.

The following definitions are also used in the Examples that follow:



EXAMPLE 38

Preparation of *N*-prop-2-yn-1-ylacetamide (i-1)

Acetyl chloride (0.52 mL, 7.3 mmol) was added to a stirred solution of propargylamine (0.5 mL, 7.3 mmol) and dimethylaminopyridine (18 mg, 0.14 mmol) in pyridine (2.5 mL) at 0°C, and the resulting mixture was allowed to warm to ambient temperature. After approximately 15 h, the reaction mixture was diluted with ethyl acetate and washed successively with 1N HCl and brine. The organic phase was dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford the title compound (i-1), which was used without further purification.

EXAMPLE 39

Preparation of *N*-prop-2-yn-1-ylbenzenesulfonamide (i-2)

Benzene sulfonyl chloride (1.16 mL, 9.1 mmol) was added to stirred solution of propargylamine (0.62 mL, 9.1 mmol) and dimethylaminopyridine (22 mg, 0.18 mmol) in pyridine (5 mL) at room temperature. The resulting solution was aged

at ambient temperature for approximately 15 h. The reaction mixture was diluted with ethyl acetate and washed successively with 1N HCl and brine. The organic phase was dried (Na_2SO_4), filtered and concentrated *in vacuo* to furnish the title compound (i-2), which was used without further purification.

EXAMPLE 40

Preparation of *N*,*N*-Dimethyl-*N*'-prop-2-yn-1-ylurea (i-3)

Dimethyl carbamylchloride (0.84 mL, 9.1 mmol) was added to a stirred solution of propargylamine (0.62 mL, 9.1 mmol) and dimethylaminopyridine (22 mg, 0.18 mmol) in pyridine (5 mL) at room temperature. The resulting suspension was stirred at ambient temperature for approximately 15 h. The reaction mixture was diluted with ethyl acetate and washed successively with 1N HCl and brine. The organic phase was dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford the title compound (i-3), which was used without further purification.

EXAMPLE 41

Preparation of *N*-Methyl-*N*-prop-2-yn-1-ylmethanesulfonamide (i-4)

Methansulfonylchloride (1.12 mL, 14.5 mmol) was added to a stirred solution of *N*-methylpropargylamine (1.22 mL, 14.5 mmol) and dimethylaminopyridine (35 mg, 0.30 mmol) in pyridine (10 mL) at room temperature. After aging for approximately 15 h, the reaction mixture was poured into ethyl acetate and washed successively with 1N HCl and brine. The organic phase was dried (Na_2SO_4), filtered and concentrated *in vacuo*, to afford the title compound (i-4), which was used without further purification.

EXAMPLE 42

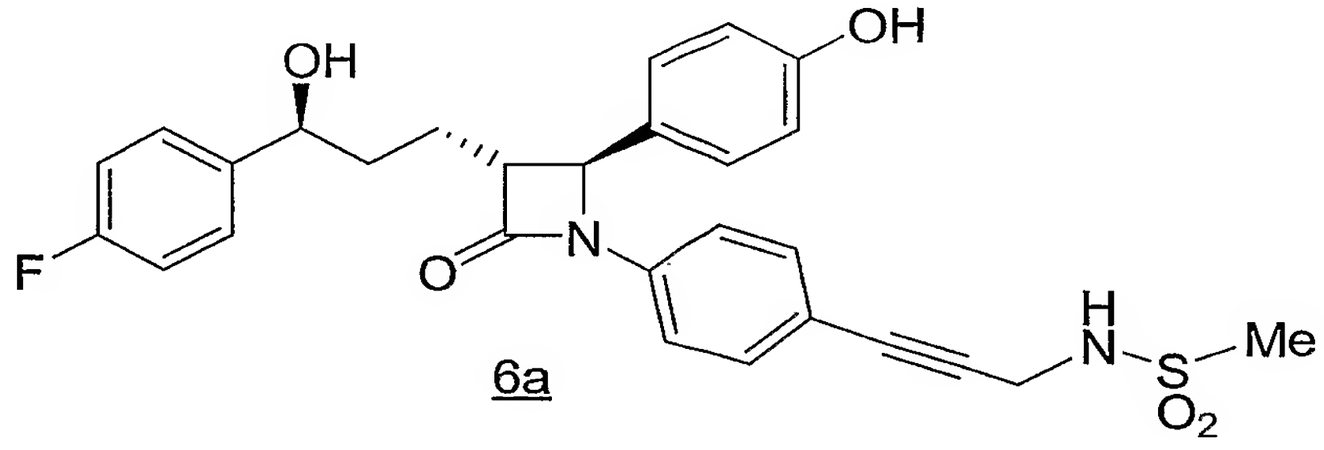
Preparation of *N*-prop-2-yn-1-ylmethanesulfonamide (i-5)

Methansulfonylchloride (1.40 mL, 18.1 mmol) was added dropwise to a stirred solution of propargylamine (1.00 g, 18.1 mmol) and dimethylaminopyridine (44.0 mg, 0.36 mmol) in pyridine (10 mL) at 0 °C. After aging for approximately 15 h, the reaction mixture was poured into 1N HCl and extracted twice with ethyl acetate. The combined organic extracts were washed with saturated aqueous sodium bicarbonate, brine, dried ($MgSO_4$), filtered and concentrated *in vacuo*, to afford the

title compound i-5. Crude i-5 crystallized on standing and was used without further purification.

EXAMPLE 43

Preparation of *N*-(3-{4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-2-(4-hydroxyphenyl)-4-oxoazetidin-1-yl]phenyl}prop-2-yn-1-yl)methanesulfonamide (Compound 6a)

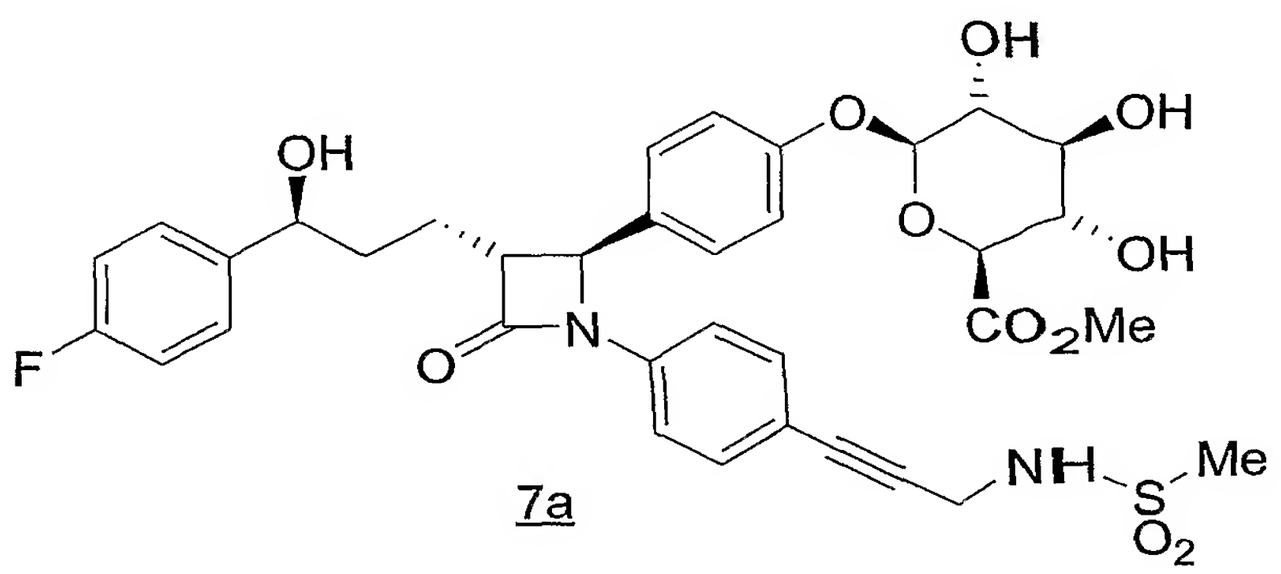


6a

Triethylamine (7 equivalents) is added to a solution of (3*R*,4*S*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-1-(4-iodophenyl)azetidin-2-one (i-6) (1.00 equivalent), *N*-prop-2-yn-1-ylmethanesulfonamide (i-5) (1.50 equivalents), tetrakistriphenylphosphine palladium(0) (0.15 equivalents) and copper(I) iodide (0.30 equivalents) in DMF (0.1 M concentration with respect to final product) under a nitrogen atmosphere and the resulting solution aged at room temperature. After completion of reaction, the volatiles are evaporated in vacuo and the crude residue can be purified by flash chromatography on silica gel to afford the title compound.

EXAMPLE 44

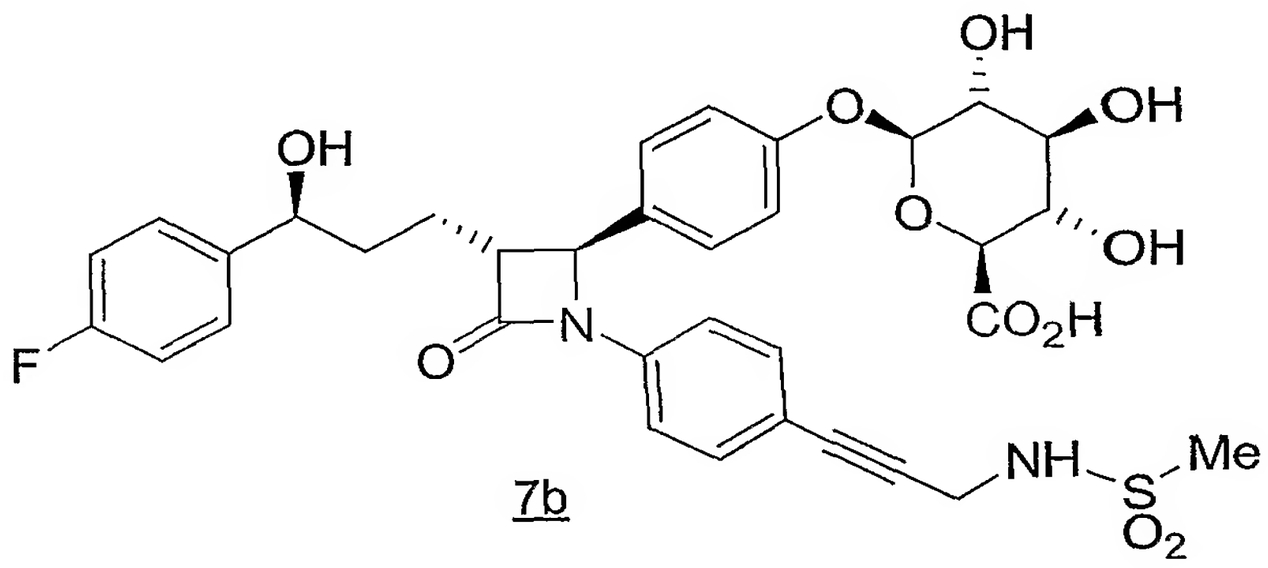
Step A: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate (Compound 7a)



7a

Triethylamine (0.07 mL, 0.502 mmol) was added to a stirred solution of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-iodophenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate (i-7) (0.050 g, 0.071 mmol), *N*-prop-2-yn-1-ylmethanesulfonamide (i-5) (0.014 g, 0.105 mmol), tetrakistriphenylphosphine palladium(0) (0.012 g, 0.010 mmol) and copper iodide (0.005 g, 0.026 mmol) in DMF (0.5 mL) under a nitrogen atmosphere and the resulting solution aged at room temperature for 18 h. The volatiles were evaporated in vacuo and the crude residue purified by flash chromatography on silica gel (gradient elution; 0-25% methanol/methylene chloride as eluent) to afford the title compound; *m/z* (ES) 713 (MH^+), 505.

Step B: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl β-D-glucopyranosiduronic acid (Compound 7b, also referred to herein as compound **2**)

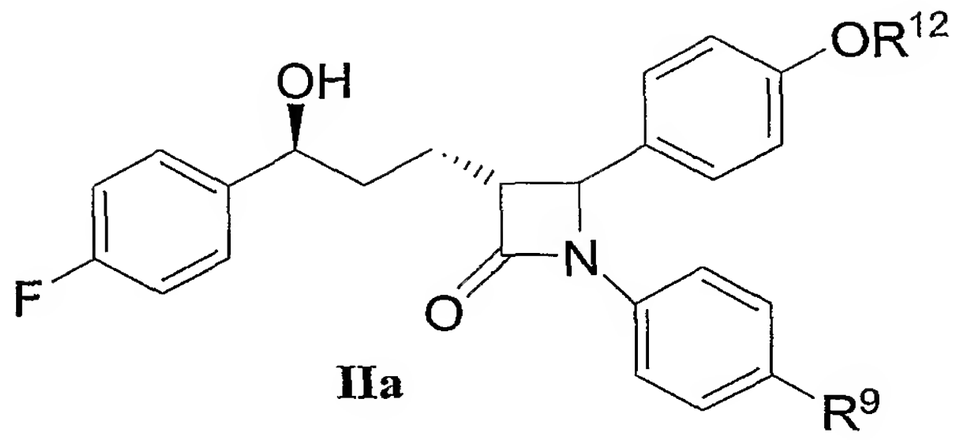


7b

A solution of compound 7a in methanol/water/triethylamine (1:7:2; 1 mL) was stirred at room temperature for approximately 1.5 h. The volatiles were evaporated *in vacuo* and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-65% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (7b); *m/z* (ES) 699 (MH^+), 505; HRMS (ES) *m/z* calcd for $C_{34}H_{36}FN_2O_{11}S$ (MH^+) 699.2024, found 699.2016.

EXAMPLE 45 (COMPOUNDS 6B TO 6G AND 7C TO 7N)

The following compounds of Formula IIa have been prepared (as indicated by MS data provided) or can be prepared using the general synthetic procedures described in Example 43 (shown in Table 13) or Example 44 (shown in Table 14).



IIa

TABLE 13

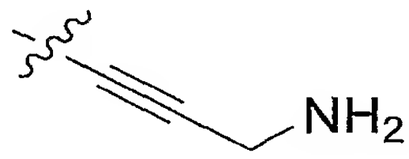
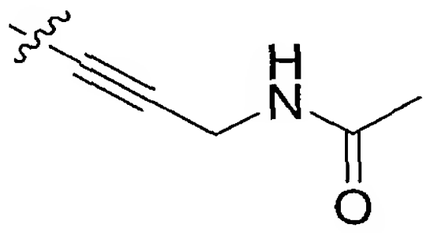
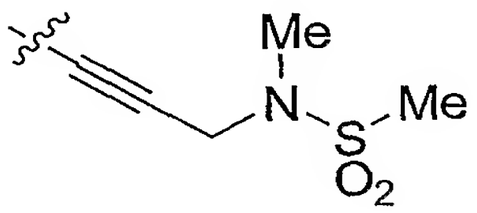
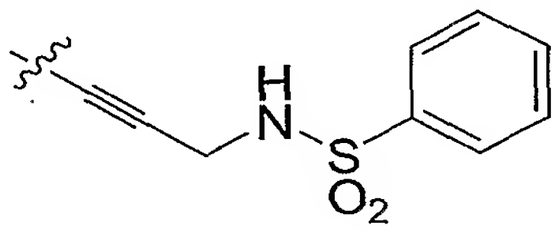
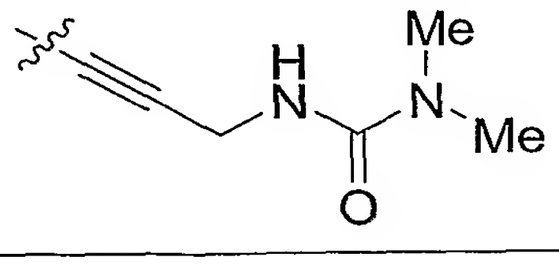
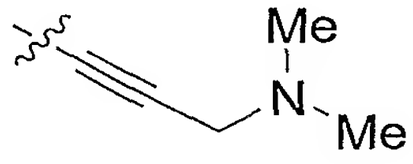
Compound	R^{12}	R^9
6b)	H	
6c)	H	
6d)	H	
6e)	H	
6f)	H	
6g)	H	

TABLE 14

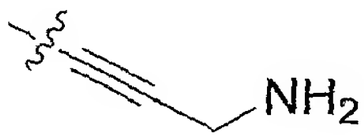
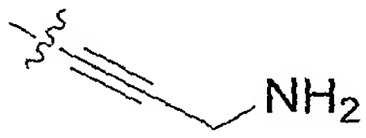
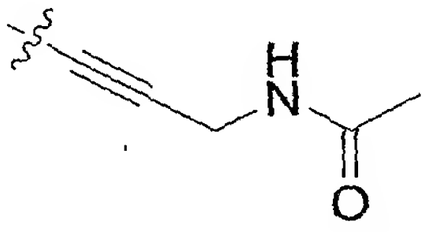
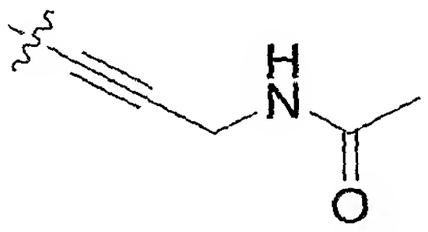
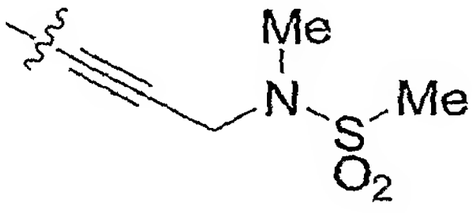
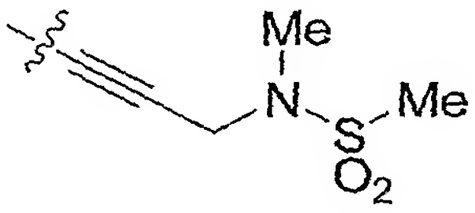
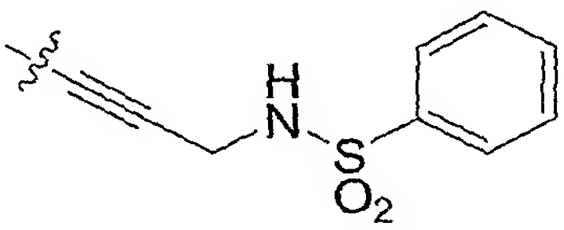
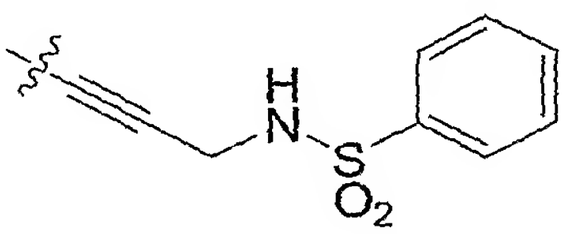
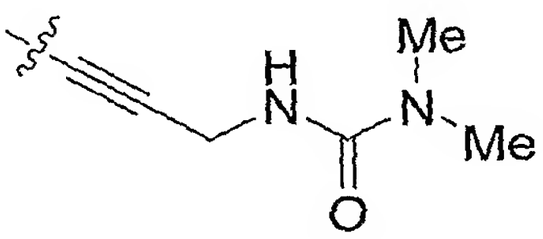
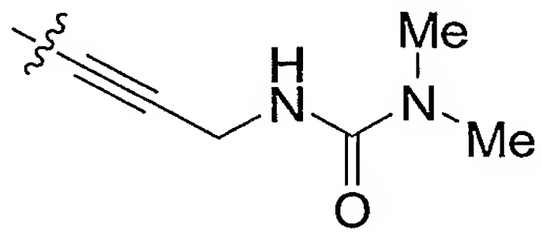
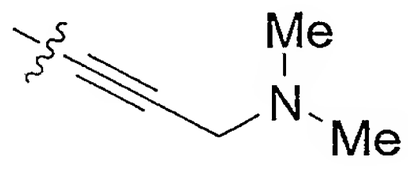
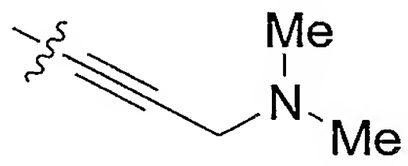
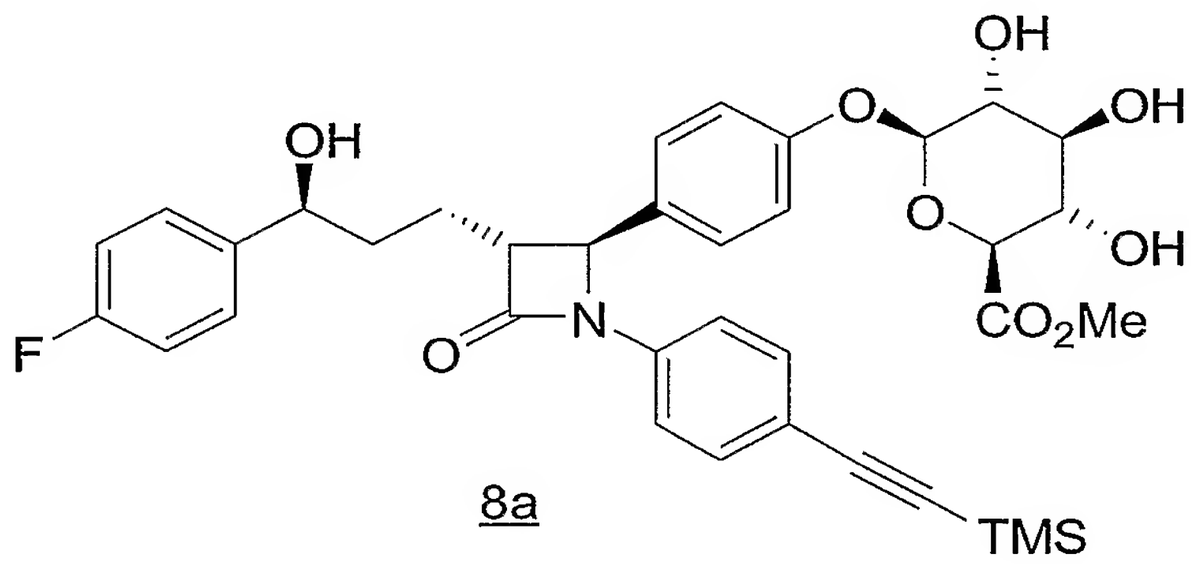
Compound	R12	R9	*m/z* (ES)	HRMS *m/z* (ES)
7c)	methyl ester glucuronide		658 (MNa^+)	
7d)	glucuronide		621 (MH^+)	(MH^+) Calcd 621.2249 Found 621.2223
7e)	methyl ester glucuronide		677 (MH^+)	
7f)	glucuronide		663 (MH^+)	(MH^+) Calcd 663.2354 Found 663.2331
7g)	methyl ester glucuronide		749 (MNa^+)	
7h)	glucuronide		735 (MNa^+)	(MH^+) Calcd 713.2180 Found 713.2170
7i)	methyl ester glucuronide		797 (MNa^+)	
7j)	glucuronide		783 (MNa^+)	(MH^+) Calcd 761.2180 Found 761.2193
7k)	methyl ester glucuronide		706 (MH^+)	

TABLE 14

Compound	R12	R9	*m/z* (ES)	HRMS *m/z* (ES)
7l)	glucuronide		692 (MH^+)	(MH^+) Calcd 692.2620 Found 692.2618
7m)	methyl ester glucuronide		663 (MH^+)	
7n)	glucuronide		649 (MH^+)	(MH^+) Calcd 649.2562 Found 649.2532

EXAMPLE 46

Step A: Preparation of 4-((2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-oxo-1-{4-[(trimethylsilyl)ethynyl]phenyl}azetidin-2-yl)phenyl methyl β-D-glucopyranosiduronate (8a)

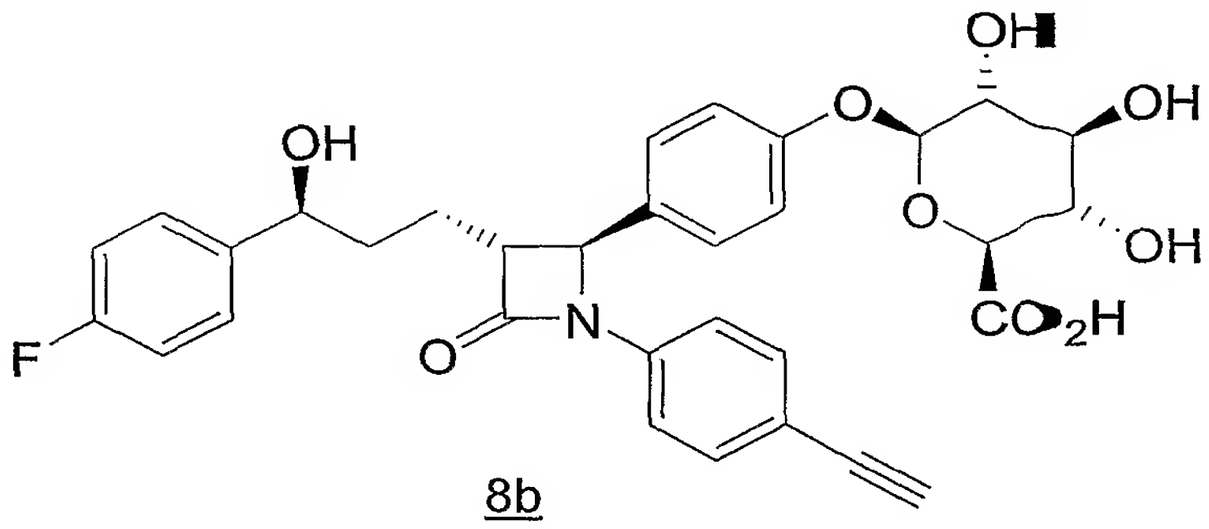


8a

Triethylamine (69.0 μL, 0.495 mmol) was added to a stirred solution of i-7 (50.0 mg, 0.071 mmol), trimethylsilyacetylene (12.0 μL, 0.085 mmol), tetrakistriphenylphosphine palladium(0) (13.0 mg, 0.011 mmol) and copper iodide (5.10 mg, 0.028 mmol) in DMF (0.5 mL) under a nitrogen atmosphere and the resulting solution aged at room temperature for 18 h. The volatiles were evaporated in vacuo and the crude residue purified by flash chromatography on silica gel

(gradient elution; 0-25% methanol/methylene chloride as eluent) to afford the title compound (8a); *m/z* (ES) 660 $(M\text{-}OH)^+$, 470.

Step B: Preparation of 4-{(2*S*,3*R*)-1-(4-ethynylphenyl)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-oxoazetidin-2-yl}phenyl β-D-glucopyranosiduronic acid (8b)

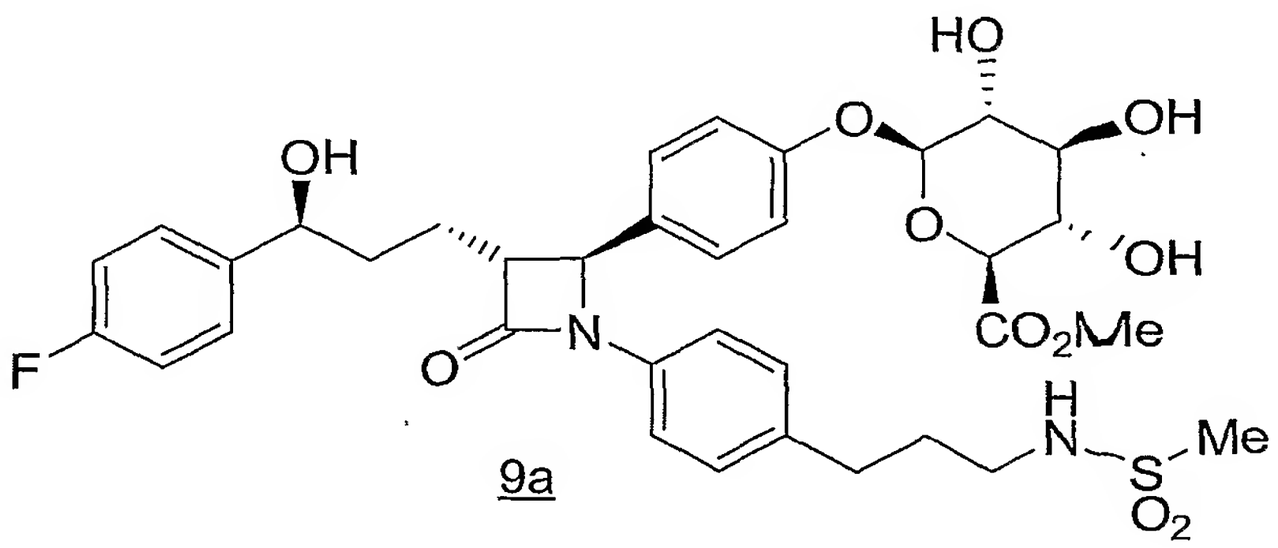


8b

A solution of 8a in methanol/water/triethylamine (0.25 mL:1.10 mL:0.40 mL) was stirred at room temperature for approximately 6 h. The volatiles were evaporated *in vacuo* and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-65% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (8b); *m/z* (ES) 574 $(M\text{-}OH)^+$, 398; HRMS (ES) *m/z* calc'd for $C_{32}H_{31}FNO_9$ (MH^+) 592.1983, found 592.1985.

EXAMPLE 47

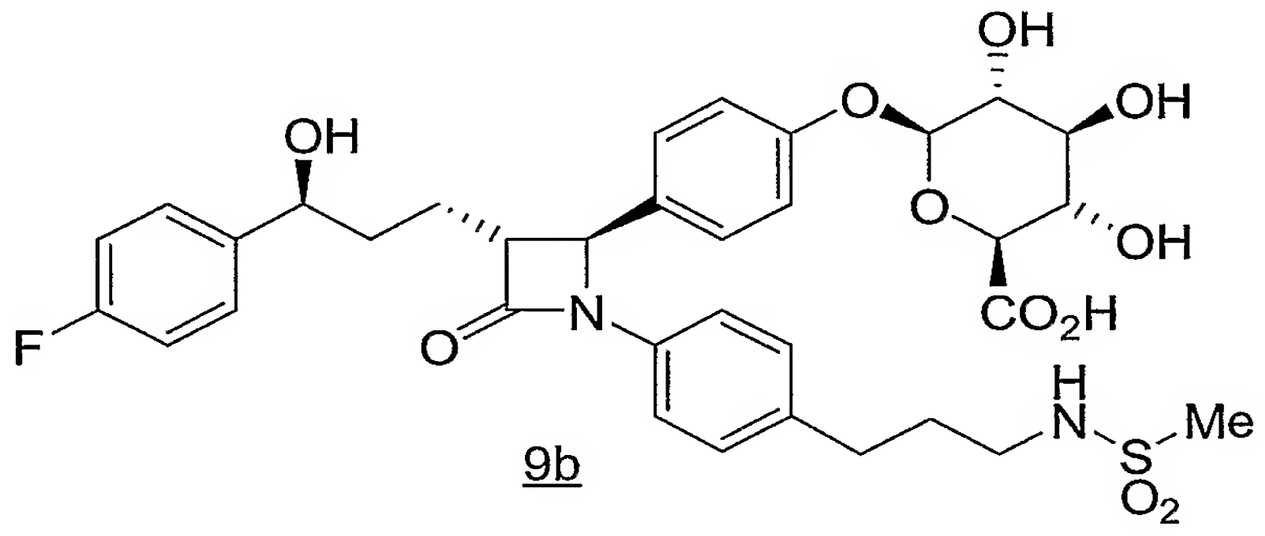
Step A: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]propyl}phenyl)-4-oxoazetidin-2-yl]phenyl methyl β-D-glucopyranosiduronate (9a)



9a

A mixture of 7a (40.0 mg, 0.056 mmol) and palladium (~8 mg of 10 wt. % (dry basis) on activated carbon) in methanol (2 mL) was hydrogenated at atmospheric pressure for approximately 1 h. The reaction mixture was filtered through a short plug of celite, eluting copiously with methanol, and the filtrate evaporated *in vacuo* to afford the title compound (9a); *m/z* (ES) 509 $(M\text{-sugar-OH})^+$.

Step B: Preparation of 4-[(2*S*,3*R*)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]propyl}phenyl)-4-oxoazetidin-2-yl]phenyl β-D-glucopyranosiduronic acid (9b)

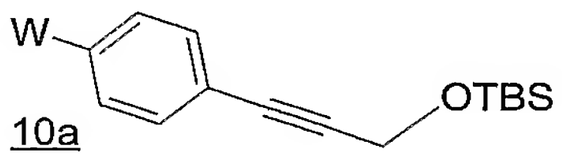


9b

A solution of 9a in methanol/water/triethylamine (1:7:2, 1 mL) was stirred at room temperature for approximately 1 h. The volatiles were evaporated *in vacuo* and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-65% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (9b); *m/z* (ES) 735 $(M+Na)^+$, 685 $(M\text{-OH})^+$, 509 $(M\text{-sugar-OH})^+$; HRMS (ES) *m/z* calc'd for $C_{34}H_{39}FN_2O_{11}S$ (MH^+) 703.2337, found 703.2337.

EXAMPLE 48

Step A: Preparation of 4-{(2*S*,3*R*)-3-[(3*S*)-3-acetoxy)-3-(4-fluorophenyl)propyl]-1-[4-(3-{[*tert*-butyl(dimethylsilyl]oxy}prop-1-yn-1-yl)phenyl]-4-oxoazetidin-2-yl}phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (10a)

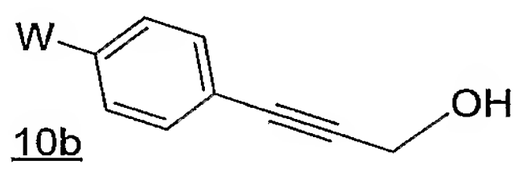


10a

Triethylamine (170 μL, 1.25 mmol) was added to a solution of i-8 (156 mg, 0.178 mmol), *tert*-butyldimethyl(2-propynyloxy)silane (43.0 μL, 0.214 mmol),

dichlorobistriphenylphosphine palladium(II) (12.0 mg, 0.018 mmol) and copper iodide (7.00 mg, 0.036 mmol) in DMF (1.3 mL) under a nitrogen atmosphere and the resulting solution aged at room temperature for approximately 20 h. The reaction mixture was poured into saturated aqueous sodium bicarbonate and extracted twice with diethyl ether. The combined organic extracts were washed with water, brine, dried ($MgSO_4$), filtered and the filtrate concentrated *in vacuo*. Purification of the crude residue by flash chromatography on silica gel (gradient elution; 15-40% ethyl acetate/hexanes as eluent) afforded the title compound 10a.

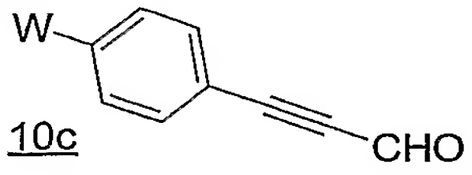
Step B: Preparation of 4-{(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-[4-(3-hydroxyprop-1-yn-1-yl)phenyl]-4-oxoazetidin-2-yl}phenyl methyl 2,3,4-tri-*O*-acetyl-β–D-glucopyranosiduronate (10b)



10b

Tetrabutylammonium fluoride hydrate (39.0 mg, 0.148 mmol) was added to 10a (136 mg, 0.148 mmol) in tetrahydrofuran (1.5mL), and the resulting solution aged at room temperature for 1 h. The reaction mixture was poured into saturated aqueous ammonium chloride and extracted twice with ether. The combined organic extracts were washed with saturated sodium bicarbonate, brine, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification of the crude residue by flash chromatography on silica gel (50% ethyl acetate/hexanes) afforded the title compound 10b.

Step C: Preparation of 4-{(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-4-oxo-1-[4-(3-oxoprop-1-yn-1-yl)phenyl]azetidin-2-yl}phenyl methyl 2,3,4-tri-*O*-acetyl-β–D-glucopyranosiduronate (10c)

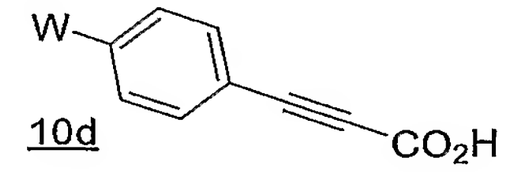


10c

Dess-Martin periodinane (33.0 mg, 0.077mmol) was added to a solution of 10b (62.0 mg, 0.077 mmol) and pyridine (31.0 μL, 0.386 mmol) in dichloromethane (1 mL) at room temperature. After 30 min, the reaction mixture was

poured into saturated aqueous sodium bicarbonate, and extracted twice with ethyl acetate. The combined organic extracts were washed with water, brine, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification of the crude residue by flash chromatography on silica gel (gradient elution; 20-40% ethyl acetate/hexanes) afforded the title compound 10c.

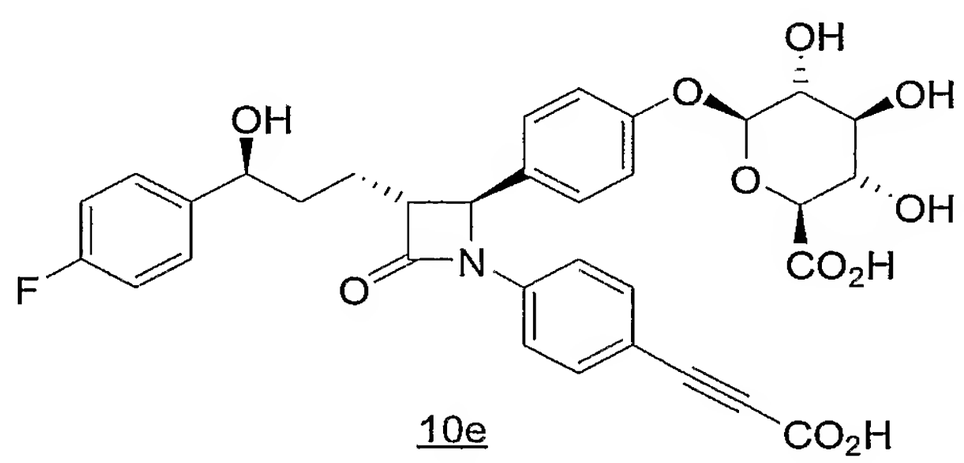
Step D: Preparation of 4-{(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-[4-(carboxyethynyl)phenyl]-4-oxoazetidin-2-yl}phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (10d)



10d

An aqueous solution (0.1 mL) of sodium dihydrogenphosphate (9.00 mg, 0.065 mmol) and sodium chlorite (5.00 mg, 0.055 mmol) was added to a solution of 10c (37.0 mg, 0.046 mmol) in *tert*-butyl alcohol (0.4 mL), dioxane (0.2 mL) and isobutylene (~0.1 mL) at room temperature. After 1.5 h, the reaction mixture was concentrated *in vacuo* and the crude residue triturated repeatedly with ethyl acetate. The organic washings were dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford the title compound 10d.

Step E: Preparation of 4-{(2*S*,3*R*)-1-[4-(carboxyethynyl)phenyl]-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-oxoazetidin-2-yl}phenyl β–D-glucopyranosiduronic acid (10e)



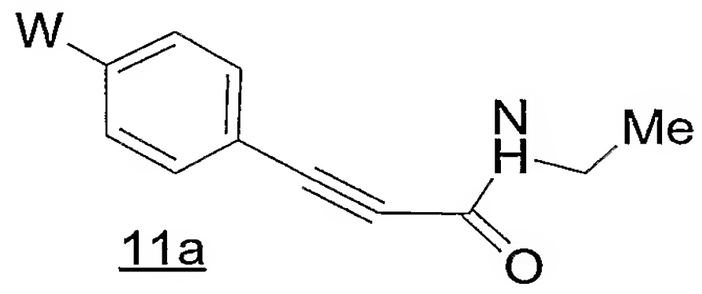
10e

A solution of 4-{(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-[4-(carboxyethynyl)phenyl]-4-oxoazetidin-2-yl}phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (10d) and sodium cyanide (~1mg, 0.020 mmol) in methanol (3mL) was heated to 45°C. After 22 h, the reaction mixture was concentrated under reduced pressure and dissolved in methanol/water/triethylamine (1:7:2, 1 mL). After stirring at room temperature for

approximately 1 h, the volatiles were evaporated *in vacuo* and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-60% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (10e), *m/z* (ES) 442.0 (M-sugar-OH)$^+$, 618.0 (M-OH)$^+$; HRMS (ES) *m/z* calcd. for $C_{33}H_{31}FNO_{11}$ (MH^+) 636.1881, found 636.1889

EXAMPLE 49

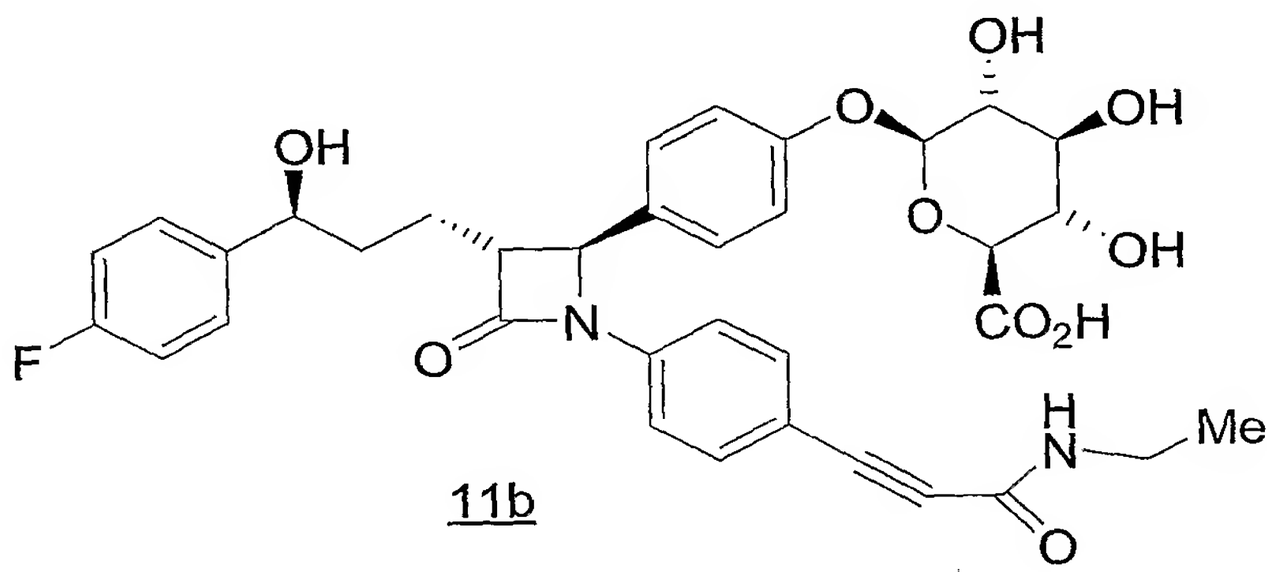
Step A: Preparation of 4-((2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-{4-(3-(ethylamino)-3-oxoprop-1-yn-1-yl]phenyl}-4-oxoazetidin-2-yl)phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (11a)



11a

A 1M solution of ethylamine hydrochloride and diisopropylethylamine in DMF (40.0 μL, 0.40 mmol) was added to 4-{(2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-[4-(carboxyethynyl)phenyl]-4-oxoazetidin-2-yl}phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (10d) (27.0 mg, 0.033 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (19.0 mg, 0.099 mmol) and 1-hydroxybenzotriazole (8.00 mg, 0.059 mmol) in DMF (0.25 mL). After 4.5 h, the reaction mixture was poured into ethyl acetate and washed successively with water and brine. The organic layer was dried, filtered and concentrated under reduced pressure. Purification of the crude residue by flash chromatography on silica gel (gradient elution; 50-60% ethyl acetate/hexanes) afforded the title compound 11a.

Step B: Preparation of 4-{(2*S*,3*R*)-1-{4-[3-(ethylamino)-3-oxoprop-1-yn-1-yl]phenyl}-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-oxoazetidin-2-yl}phenyl β-D-glucopyranosiduronic acid (11b)



11b

A solution of 4-((2*S*,3*R*)-3-[(3*S*)-3-(acetyloxy)-3-(4-fluorophenyl)propyl]-1-{4-(3-(ethylamino)-3-oxoprop-1-yn-1-yl]phenyl}-4-oxoazetidin-2-yl)phenyl methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (11a) (22.0 mg, 0.026 mmol) and sodium cyanide (~1mg, 0.020 mmol) in methanol (3mL) was heated to 45°C. After 18 h, the reaction mixture was concentrated under reduced pressure and dissolved in methanol/water/triethylamine (1:3:1, 2.5 mL). After stirring at room temperature for approximately 1 h, the volatiles were evaporated *in vacuo*, and the crude residue purified by preparative reversed phase high performance liquid chromatography on YMC Pack Pro C18 phase (gradient elution; 10-60% acetonitrile/water as eluent, 0.1% TFA modifier) to give the title compound (11b) *m/z* (ES) 663.0 $(M+H)^+$; HRMS (ES) *m/z* calcd. for $C_{35}H_{36}FN_2O_{10}$ (MH^+) 663.2354, found 663.2341.

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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Patents, patent applications, publications, product descriptions, Genbank Accession Numbers and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.